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UNIVERSITY OF ALBERTA

THE ACTIONS OF HYDROGEN SULPHIDE ON CENTRAL NERVOUS TISSUE

©

BY

SAMUEL BINAMIN KOMBIAN

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
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DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

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"Life is the art of drawing sufficient conclusions
fom insufficient premises."
Samuel Butler

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **The Actions of Hydrogen Sulphide on Central Nervous Tissue** submitted by **Samuel B. Kombian** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Pharmacology**.

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April 30, 1992

Date

To

My wife Salamatu and the kids, Yenukwa and Binyoang Hawa

ABSTRACT

Hydrogen sulfide is a toxin which produces motor disturbance, amnesia, respiratory distress, and death due to arrest of central respiratory drive. The toxic actions of sulfide were examined on neurons using neurochemical and electrophysiological techniques *in vivo*, and *in vitro*. Also, the actions of a potential antidote were examined both *in vivo* and *in vitro*.

Neurochemical studies revealed that sulfide increased the levels of excitatory and inhibitory amino acid neurotransmitters in the brainstem. Levels of all the biogenic amines increased in the brainstem, whereas only adrenaline and noradrenaline levels increased in the hippocampus and striatum after sulfide treatment.

In vivo treatment with sulfide increased the recovery of amino acids from the hippocampus and striatum. More pronounced increases occurred during local perfusion with sulfide. These regional changes did not follow the same trend as the total content changes.

In frog sympathetic ganglia, sulfide elicited a depolarization and enhanced the slow hyperpolarization synaptic responses induced by muscarine and adrenaline but not the fast depolarization response induced by nicotine. Sulfide also potentiated the electrogenic Na^+/K^+ pump.

In vitro sharp microelectrode and patch clamp intracellular recording from the dorsal raphe (DR) nucleus showed four dose-dependent postsynaptic responses in serotonergic neurons to sulfide; 14% responded with an outward current; 43% responded with a weakly voltage dependent inward current; 30% responded with a biphasic response, an inward current followed by an outward current. The hyperpolarization was due to a K^+ current which could be blocked by Ba^{2+} and Cs^+ in combination, or Cd^{2+}. The inward current did not

result from blockade of the Na^+/K^+ pump, but was reduced by Cd^{2+}. The results from the biphasic cells suggest that the outward current may depend on elevation in intracellular Ca^{2+} concentration, while the inward current may be carried by Ca^{2+}. The remaining 13% did not have a postsynaptic response to sulfide. In most responding cells removal of sulfide was followed by an Na^+/K^+ pump mediated outward current.

Sulfide also inhibited all three synaptic responses recorded in the DR but with different potencies.

The LD_{50} of sulfide in rats (15 mg/kg) was nearly doubled (26 mg/kg) by 20 min pretreatment with dithiothreitol. In contrast, dithiothreitol did not reverse sulfide toxicity. These *in vivo* actions of dithiothreitol were partially supported by *in vitro* electrophysiological studies in the DR.

Any or all of the *in vivo and in vitro* actions of sulfide on nervous tissue could account for the neurological disturbances that occur in hydrogen sulfide poisoning. The antidotal potential of dithiothreitol in acute lethal exposure is limited but it may promote rapid recovery or prevent neurological sequelae in survivors of hydrogen sulfide poisoning.

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LIST OF ABBREVIATIONS

ACh	acetylcholine
ACSF	artificial cerebrospinal fluid
AHP	afterhyperpolarization
ALA-S	delta-aminolaevulinic acid synthase
4-AP	4-aminopyridine
ALA	alanine
ASP	aspartate
ATP	adenosine 5'-triphosphate
ATPase	adenosine 5'-triphosphatase
cc	cubic centimeter
CNS	central nervous system
CsCl	cesium chloride
5-CT	5-carboxamidotryptamine
DSC	depolarizing synaptic current
DSP	depolarizing synaptic potential
DTT	dithiothreitol
E_K	potassium equilibrium potential
EC_{50}	median effective concentration
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
EPSP	excitatory postsynaptic potential
EPSC	excitatory postsynaptic current

sEPSP/EPSC	slow excitatory postsynaptic potential/current
g	gram
GABA	γ-amino butyric acid
GAD	glutamic acid decarboxylase
gK_{ATP}	ATP-sensitive potassium conductance
gK_{Ca}	Calcium-activated potassium conductance
gK_{Na}	sodium-activated potassium conductance
GLU	glutamate
GLN	glutamine
GLY	glycine
GTP	guanosine 5'-triphosphate
GΩ	gigaohm
Heme-S	heme-synthase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HS^-	hydrosulfide anion
H_2S	hydrogen sulfide
Hz	Hertz
I_A	"A" current
I_K	delayed rectifier
I_M	"M" current
I_Q	"Q" current
I_H	"H" current
I_{IR}	inward rectifying potassim current

$I_{5\text{-}CT}$	5-CT-activated potassium current
ip	intraperitoneal
I-V	current-voltage
ICV	intracerebroventricular
kg	kilogram
LD_{50}	median lethal dose
l	liter
m	milli
MAO	monoamine oxidase
$M\Omega$	megaohm
msec	millisecond(s)
ml	milliliter
min	minute(s)
mg	milligram
μ	micro
n	number
nA	nanoampere
NaHS	sodium hydrosulfide
NRC	National Research Council
osm	osmolarity
p	pico
PE	polyethelyne
pp	pages

PNMT	phenyl-ethanolamine-N-methyl transferase
sec	second(s)
SER	serine
TAU	taurine
TEA	tetraethylammonium
TTX	tetrodotoxin
V	volt(s)
V_C	command potential
V_H	holding potential
WCR	whole-cell recording
WISP	Waters Intelligent Sample Processor
$[\,]_i$	intracellular concentration
$[\,]_o$	extracellular concentration

CHAPTER I

INTRODUCTION

I. GENERAL LITERATURE REVIEW ON HYDROGEN SULFIDE TOXICITY

a. Historical Background

Hydrogen sulfide (H_2S) poisoning in humans was first described unknowingly in the 16th century when Ramazzini (1713) observed that a "volatile acid" released from excrement caused an irritation of eyes of cesspit cleaners. Putrefying organic matter is now known to be a major source of H_2S and the eye irritation test is the basis of the Threshold Limit Value (TLV) for H_2S in the work place in America.

Sometime around 1775, a Swedish chemist, Carl Wilhelm Scheele , while reacting ferrous sulfide with a mineral acid, detected the foul odour which he called "schwefelluft" (sulphur air). At about this time, numerous reports appeared about deaths due to exposure to sewer gas(es) in Paris. A report regarding these exposures by M.Halle (Mitchell and Davenport, 1924) described two distinct types of poisonings: the first type was mild and involved inflammation of the eyes and mucous membranes, with irritation, as was described by Ramazzini; the second type was more severe with fulminating asphyxia. Only recently did analyses of these sewer gases implicate H_2S as the main causative agent of those 18th century fatalities. Sublethal, but toxic, exposures to humans and farm animals occur frequently in several industrial and environmental settings. Lethal exposures occur during industrial accidents (gas well blowout) and natural disasters (e.g following volcanic eruptions). As late as the mid-1980's, toxic sulfide exposures and lethality have occurred in Alberta from gas well blow outs.

b. Physical Characteristics

H_2S is a colourless gas which in low perceptible concentration, possesses an odour much like that of rotten eggs. It is heavier than air and moderately soluble in water. It reacts with both cations and anions to produce sulfide salts and sulfides and polysulfide derivatives respectively. In aqueous solution, hydrogen sulfide dissociates into HS^- and S^{-2} with pKa values of 7.04 and 11.96 respectively. At physiological pH, (pH 7.4) H_2S exists as two-thirds (2/3) HS^- and a third as unionized soluble gas (Beauchamp *et al.*, 1984). Also, alkaline salts of sulfides generate HS- and S^{-2} in solution. The gas exhibits high solubility in lipophilic media. Important conversion factors for H_2S are: 1% volume = 10,000 ppm, 1 mg/l = 717 ppm (STP).

c. Potential Sources and Exposure Parameters of H_2S

H_2S is a common by-product of putrefaction of organic matter which contains sulfur as part of its structure. Its formation is due to incomplete oxidation of the sulfur. Toxic exposure occurs in both environmental and industrial settings where decay continuously takes place or is part of a processing step, such as in the leather tanning industry. Numerous accidental exposures occur in the oil and gas industry (petroleum prospecting and storage sites), mines, agricultural settings, and during volcanic eruptions (Milby, 1962; Beauchamp *et al.*, 1984). Because of this widespread occurrence, safety concentration ranges and their effects have been well established for most work places. Odour threshold is determined to be 0.01 parts per million (ppm), whereas life threatening amounts are in the range of 700-1400 ppm (Smith, 1983). This lethal level is several thousand times greater than the perceptive levels. This means different levels of exposure can occur without overt clinical signs or symptoms of poisoning. Exposure is therefore conventionally described, based on H_2S concentration and duration of contact with the gas; as acute, subacute and chronic.

Acute exposure usually involves a single massive concentration (>700 ppm) in less than 24 hours, onset of toxicity is usually rapid and intense. In humans and rats, acute exposure usually produces clinical signs which implicate the central nervous system (CNS), e.g convulsions, unconsciousness, respiratory distress (Milby, 1962; National Research Council report-NRC, 1979) and ultimately death. Human survivors often display various types of memory deficits. During acute exposure of laboratory animals, a quantitative parameter called the LD_{50} is commonly used to describe the level of toxicity. It is defined as the dose or concentration of compound or toxin that would be lethal to fifty percent (50%) of the population of experimental subjects and is usually expressed as mg/kg or moles/kg body weight (Smith, 1983). To perform acute toxicity studies of sulfide in animals, aqueous solutions of the alkali salts of sulfide such as sodium hydrosulfide (NaHS) and sodium sulfide (Na_2S) are injected intraperitoneally (IP). Using the solution of the salts offers several advantages over the gas (H_2S), while achieving toxic responses comparable to the gas. It has been shown that the gas and NaHS or Na_2S, once in solution or in the blood both generate HS^- and S^{-2} which then serve as the toxin (Beauchamp *et al.*, 1983). Also, it has been determined that the systemic effects due to the gas and the salts are identical except for airway effects, which is been attributed to the local irritant effect of the gas on airway mucosa (Lopez *et al.*, 1989; Reiffenstein *et al.*, 1988). Furthermore, exposure to the salts leads to brain levels of sulfide similar to those attained with inhalation of the gas (Reiffenstein *et al.*, 1988; Lopez *et al.*, 1989). A notable advantage of using the salts over the gas is the easy handling of the salts and therefore a lower risk to the experimenter. Also, it is much easier to control the dose of the salt than it is to control the dose of the gas. Also, no expensive and sophisticated equipment is required to administer and control the salts as is the case with the gas. Finally, it has been shown that the salts have the added advantage of producing toxic effects much more rapidly than does the gas, thus reducing the length of time between exposure and death (Beauchamp *et al.*, 1984; Smith, 1983).

In subacute exposure, H_2S concentration is usually low (50-100 ppm) but occurs over a longer period of time, several minutes to hours with milder symptoms such as eye and throat irritation (Milby, 1962; NRC, 1979). Biochemical alterations in several enzymes and membrane constituents have been observed following such low level exposures. Chronic exposure in humans may occur but is difficult to define clinically since the body is capable of rapidly detoxifying minute, innocuous amounts of sulfide that may be encountered daily ($<$ 30 ppm a day over several days or even years; Haggard, 1921; Evans, 1967). The likelihood of sulfide accumulating to dangerous levels is therefore very low (Bittersohl, 1976; Haggard, 1925). However, it has been suggested that repeated exposure over long periods may lead to such symptoms as neurasthenia in humans (Ahlborg, 1951; Illinois Institute for Environmental Quality IIEQ, 1974), alteration in brain metabolism (Savolainen *et al.*, 1980; Haiden *et al.*, 1980) and some reproductive effects (Barilyak *et al.*, 1975; Hannah *et al.*, 1989a; Roth, 1989) in experimental animals.

As indicated above, with most toxic exposures, the effects are dependent on both the duration and intensity of the exposure (Milby, 1962; Smith, 1983). O'Donoghue (1961) reported that H_2S toxicity was related to the concentration of exposure only. In contrast, Clanachan (1979) showed that at higher concentration of H_2S, the time required to produce unconsciousness and death in mice was shorter as compared to the lower concentration; thus suggesting that acute H_2S intoxication was also time-dependent. This discord may be due to the fact that in the former study, whereas the rate of increase of H_2S concentration influenced the toxicity, the time-concentration relationship of exposure on lethality was not critically examined (Clanachan, 1979). In humans, it is known that death can occur from exposure to 100 ppm H_2S for a 8-48 hours or 1000 ppm for a few minutes (NRC-Canada, 1981). More studies are required to examine more critically, the relationship between duration of exposure and concentration of H_2S on biological systems. At the present, because of the time factor (which can vary widely) involved in the classification of sulfide toxicity with the qualitative terms above, it is better to describe sulfide intoxication in terms of the clinical symptoms the victim presents, which better reflects the magnitude of the intoxication.

d. Absorption, Distribution, Metabolism and Elimination of H_2S.

Most exposures of humans to H_2S is often as a result of an environmental catastrophe or industrial accident and thus hydrogen sulfide gas is the main form of poison. The major route of access to the systemic system is therefore through absorption of the gas through the mucosa of the lungs, with very little absorption through intact skin (Patty, 1949; Milby, 1962; Smith, 1983; Beauchamp *et al.*, 1984). The first line of defense during exposure is therefore the airway linings of the respiratory tract which may sustain substantial damage (Lopez *et al.*, 1988; Hulbert *et al.*, 1988).

Following absorption from the respiratory tract, sulfide enters the blood stream where metabolic processes are rapidly engaged to detoxify it (Vigil, 1979). If the latter process is overwhelmed by massive absorption, large amounts get distributed to other organs, especially the brain. In the brain, the preferred regional distribution pattern seems to follow that of increased lipid content and solubility since sulfide is very lipophilic. For example, higher levels of sulfide are found in the brainstem and cerebellum, structures with higher lipid content than are found in the hippocampus of rats injected (ip) with NaHS (Warenycia *et al.*, 1988). The determination of this preferred distribution of sulfide to highly lipophilic regions has led to the choice of the brain stem as the structure to examine for changes in sulfide levels in forensic cases. The brainstem of victims of sulfide intoxication demonstrate significant increases in sulfide content as compared to controls (Warenycia *et al.*, 1989).

Studies in rats, dogs and other animals have shown that the major organ involved in metabolism and therefore detoxification of sulfide is the liver and to a lesser extent, tissues such as the kidney, and lungs. H_2S also interacts with oxygenated hemoglobin (Dennis and Read, 1927; Patty, 1963; Vigil, 1979) to produce less toxic oxidised products. The main products of metabolism are polysulfides, polythionates, thiosulfates and sulfates. Of these, sulfate is the major metabolite and is usually found in urine between 6-12 hours post exposure (Gunina, 1957; Curtis *et al.*, 1972; Beauchamp *et al.*, 1984; Smith *et al.*, 1983). The mechanism(s) by which these conversions occur are not exactly known although it is believed to be by both enzymatic (sulfide oxidase) and non enzymatic means. Regardless of the mechanism, H_2S biotransformation occurs rapidly and only an absorption rate that exceeds the rate of detoxification or oxidation can lead to systemic intoxication (Vigil, 1979). The major route of elimination is via the kidneys in urine, with some elimination from the lungs as unchanged sulfide gas.

e. Clinical Manifestation of Sulfide Poisoning in Humans and Animals.

H_2S is a moderately soluble gas and because of its route of intake (inhalation), it is rapidly absorbed leading to high concentrations of unoxidised gas in the blood stream. When exposure levels in humans and animals exceed 500 to 700 ppm, systemic intoxication predominates (Milby, 1962; NRC-Canada, 1979) and the signs and symptoms are clear manifestations of CNS toxicity (Haggard, 1925). Clinical signs of acute systemic intoxication include sudden fatigue, dizziness, intense anxiety, convulsions, loss of olfactory function, unconsciousness, collapse, respiratory and cardiac failure leading to death (Ahlborg, 1951; Mitchell and Yant, 1925). Moderately high concentrations of H_2S stimulates the respiratory system (Haggard *et al.*, 1922) by a peripheral mechanism via stimulation of chemoreceptors in the carotid body (Smith, 1989). This effect is transient and results in hyperpnoea (leading to increased intake of gas) and occasionally, bradycardia. Although this peripheral effect is known to occur, respiratory arrest and asphyxia are believed to be responsible for lethality following sulfide intoxication and are thought to be due to paralysis of brainstem respiratory centres (Henderson and Haggard, 1943; Milby, 1962; Beauchamp *et al.*, 1984). Non-fatal systemic intoxication is usually followed by rapid and complete recovery with very little sequelae (McCabe and Clayton, 1952; Ahlborg, 1951; Milby, 1962). If they do occur, they are usually due to CNS damage during the time of collapse and also the accompanying anoxia due to the H_2S-induced asphyxia (Milby, 1962). Sequelae are generally neurasthenic in nature and may last for days or months (Poda, 1966; Ahlborg, 1951) with symptoms such as fatigue, drowsiness, headaches, lack of initiative, nausea, nervousness, irritability, anxiety, depression, poor memory, decreased libido and some otoneurological symptoms and disturbance in equilibrium (Report on H_2S toxicity, 1988). Whereas the above effects may represent reversible changes in various regions of the CNS, significant brain damage has been reported in both animals and humans exposed to levels greater than 500 ppm of H_2S (Report on H_2S toxicity, 1988). For example, exposure to 500 ppm of H_2S for 22 minutes has been shown to cause necrosis of cerebral cortical cells, reduction in purkinje cells of the cerebellum and focal gliosis in the brain of rhesus monkeys (Lund and Wieland, 1966; Beauchamp *et al.*, 1984).

In subacute exposure, that is between 100 to 500 ppm, irritation of mucous membranes of the eyes and respiratory tract predominate (Milby, 1982). Prolonged exposure to these concentrations of H_2S causes rhinitis, pharyngitis, bronchitis and pneumonia and because of its ability to penetrate deep into the lungs, it may also produce inflammation of the alveoli leading to pulmonary edema (Ahlborg, 1951; McCabe and Clayton, 1952; Sollmann, 1948; Milby, 1962). CNS effects may also occur during subacute exposure including headaches, fatigue, irritability, insomnia and depression (Milby, 1962). Behavioral studies in rats exposed to these levels of H_2S revealed significant inhibition of discriminated avoidance response at a rate and intensity proportional to the concentration of H_2S used (Higuchi, 1971). During chronic H_2S exposure resulting from repeated encounters of for example below 100 ppm, the clinical manifestations are usually neurasthenic in nature and include symptoms such as lethargy, dizziness, loss of appetite, fatigue, headache, mental depression, irritability, poor memory, abnormal peripheral reflexes and gastrointestinal disturbances (Ahlborg, 1951; Rubin and Arieff, 1945; Report on H_2S toxicity, 1988). Ahlborg however, cautions that these may be sequelae of previous exposure to much higher levels. It was further pointed out that there was the difficulty of determining if these symptoms were actually attributable to H_2S exposure or to the "psychic strain" of working in an environment with known risks of H_2S exposure (Ahlborg, 1951; Vigil, 1979).

f. Structural and Functional Studies in Animals: *The Respiratory System*.

The effects of H_2S on microanatomical structure and function of the respiratory tract have been examined, especially following low level exposures over several hours (subacute). Lopez (1990) demonstrated that, exposure of rats to H_2S at concentrations between 200-400 ppm for 4 consecutive hours caused severe, but reversible (within 44 hours) injury to ciliated epithelium of the nasal cavity. In this same study, he also showed that there was a moderate and transient inflammatory response in the alveolar regions of the lungs. He further reported a severe but transient pulmonary oedema which was characterised by notable transudation of fluid into the bronchoalveolar space. Complete reversal of this effect occurred within 44 hours. Finally, he reported a severe irreversible necrosis of olfactory cells. In this study, no effect was seen in the morphology of the alveolar epithelium, the basal lamina nor the pneumocytes. In another study on airway systems in the guinea pig, Hulbert *et al.* (1989) observed an increase in baseline airway calibre when guinea pigs were exposed to 100 ppm of H_2S for 1 hour. They hypothesized that this effect was due to either a blockade of cholinergic tone (bronchodilation) or an enhancement of the sympathetic tone through increase in circulating catecholamine levels. In sub-acute studies, Hulbert *et al.* (1989) observed a proliferation of ciliated and basal cells of rat lung tissue with a decline in non-ciliated clara cells following exposure to 100 ppm of H_2S for 8 hours a day and 5 days a week for 5 weeks. In contrast to the results obtained from the guinea pig experiments, these rats showed "bronchial hyper-reactivity" to methacholine aerosol challenges. The underlying mechanism of this hyper-reactivity is not known (Hulbert *et al.*, 1989).

g. Biochemical Studies

Biochemical alterations in heme-synthesizing enzymes such as δ-aminolaevulinic acid synthase (ALA-S) and haem-synthase have been reported in workers exposed to minute amounts of H_2S and methylmercaptan (Jappinen, 1990; Tenhunen *et al.*, 1983). However, others could not find significant changes in several enzymes in the erythrocytes of rats and mice exposed to sublethal concentrations of H_2S (Khan *et al.*, 1990). Furthermore, only small decreases in enzymes, such as superoxide dismutase and alkaline phosphatase could be found in the erythrocytes of rats exposed to higher concentrations (Khan *et al.*, 1990; Lopez *et al.*, 1987). In the brain, subacute exposure leads to a cumulative decrease in orotic acid uptake and cytochrome c oxidase activity, with little change in protein, glutathione and ribonucleic acid (RNA) contents, and acid protease, superoxide dismutase and actylcholinesterase activity (Sovolainen *et al.*, 1980). Selective reduction of cytochrome oxidase (Hayden, 1988) and elevations in alkaline phosphatase (Hayden, *et al.*, 1987) have been reported in fetal rat brains following exposure. Also, levels of putative amino acid neurotransmitters such as glutamate, γ-amino butyric acid (GABA) and aspartate have been shown to decrease following chronic low level exposure to H_2S (Hannah *et al.*, 1988; Hannah, *et al.*, 1989c).

h. The Search for the Mechanism of Hydrogen Sulfide Toxicity and Treatment.

The observation in 1863 by Hoppe-Seyler of the effects of H_2S on blood pigments led to the unfortunate, false hypothesis that sulfide was a blood poison, analogous to carbon monoxide, the mechanism of toxicity of which had been well elucidated by Claude Barnard (1865). This led to several years of research focusing mainly in the area of blood. Another early observation regarding the toxicity of sulfide was the finding by Corneille and Heymans that sulfide, cyanide and azide all stimulated chemoreceptors in the carotid body (Smith, 1989). A large body of evidence soon established that even though sulfide and cyanide both stimulated the carotid body, lethality due to sulfide was through paralysis of respiration, presumably through an effect on the respiratory centre in the brainstem (Henderson and Haggard, 1943; Evans,1967; Milby,1962; Amman,1986). In the latter part of the 20th century, biochemical evidence started emerging about the biological effects of cyanide, azide and sulfide. They all inhibit cytochrome oxidase (an enzyme important in mitochondrial metabolism and therefore the generation of metabolic energy; Poyton *et al.*, 1988) *in vitro* (Smith *et al.*, 1977; Nicholls, 1975). Sulfide has been demonstrated to be more potent than cyanide in the inhibition of this enzyme (Nicholls *et al.*, 1975) although they possess an identical LD_{50} (Persson *et al.*, 1985; Warenycia *et al.*, 1988). It has however, been difficult to demonstrate inhibition of this enzyme *in vivo* and hence to associate the lethality to this effect (Smith *et al.*, 1977). This has been recently demonstrated *in situ* for cyanide but not for H_2S (Piantadosi and Sylvia, 1984). Using particulate preparation of cytochrome oxidase from beef heart, Smith *et al.*, (1977) demonstrated that undissociated H_2S was a more potent inhibitor of this enzyme than it's ionic form (HS_-).

Protective studies following H_2S intoxication have been attempted without much success. Even though victims of sulfide intoxication respond to hyperbaric oxygen (Milby, 1962; Ahlborg, 1951), oxygen inhalation did not significantly improve sodium sulfide-induced mortality (Smith *et al.*, 1976). The former finding lent credence to the hypothesis that acute toxicity of H_2S was due to inhibition of cytochrome oxidase. However, the very rapid manner in which acute sulfide exposure produces toxic manifestations makes inhibition of cytochrome oxidase most unlikely to be the mechanism by which sulfide produces its toxicity/lethality. Furthermore, the signs and symptoms observed during poisoning could not be solely accounted for by this mechanism.

Also, based on the numerous similarities in toxicity profiles of H_2S and cyanide (see above) and the well established ability to prevent cyanide fatality by induction of methemoglobinemia with nitrite, this agent was tried in sulfide poisoning without much success (Smith and Gosselin, 1964). These authors interpreted the lack of overwhelming success of this treatment to reverse sulfide toxicity to be due mainly to the rapid nature of sulfide action. Furthermore, the induction of methemoglobinemia with sodium nitrite or p-amino propiophenone prior to sulfide application provided only partial protection to armadillos, mice and rabbits (Smith and Gosselin, 1964). Methemoglobin has been shown to restore the cytochrome oxidase activity of erythrocytes after it had been inhibited by sodium sulfide (Smith *et al.*, 1977). Oxidised, but not reduced, glutathione protected against sodium sulfide lethality only when given prior to exposure (Smith and Abbanat, 1966). In the same study, it was further shown that oxidised glutathione and methemoglobin produced an additive protective effect when used together. The proposed mechanism of action was that of scavenging: interacting with free sulfide ions before the latter interacted with the vital enzymes (Smith and Abbanat, 1966; Beauchamp *et al.*, 1984).

With time, and the apparent lack of success in producing effective antidotes to sulfide intoxication (see above) and also, the lack of convincing evidence to correlate the very rapid nature of the acute systemic sulfide intoxication to the relatively slow process of metabolic uncoupling by cytochrome oxidase inhibition, people begun to ask different questions in an effort to explain the very rapid nature of the lethality.

Following acute injection of sodium hydrosulfide into rats, several signs of central nervous system (CNS) disturbance are observed prior to death. These range from motor inco-ordination and convulsion to respiratory and cardiovascular distress (Smith and Gosselin, 1979). The observation that death was ultimately due to paralysis of central respiratory drive was made quite early (Henderson and Haggard, 1943; Milby, 1962, Evans, 1967). So, after the inability of the metabolic uncoupling hypothesis (even within the CNS) to explain the rapid toxicity of sulfide, much attention was turned to other aspects of CNS function. It was mainly the enzymatic alterations produced by sulfide in the brain that received most attention. Sulfide has thus been shown to inhibit alkaline phosphatases and adenosine triphosphatase

(ATPase) enzymes in cerebellar cortical tissues (Kosmider and Zajusz, 1966; Beauchamp *et al.*, 1984), cerebral cortical cytochrome oxidase (Savolainen *et al.*, 1980), and acetyl- cholinesterase (Maneckjee, 1985). From these findings, it was suggested that the effects of H_2S on CNS may be due to inhibition of enzymes in the CNS including those of the respiratory centre. As McLardy (1970) had observed sulfide loading into synaptic vesicles containing zinc, it was proposed that this inhibition was as a result of sulfide binding to metal ions which serve as co-factors for those enzymes (Beauchamp *et al.*, 1984). However, the questions that arise from all such findings are: *1*. are the enzyme inhibitions the final or terminal events underlying the toxicity or do they merely serve as a means to an end? *2*. are there other effects of sulfide on neuronal properties and function that may be more important targets or substrates for sulfide toxicity? *3*. Finally, can any or all of these events occur fast enough to explain the rapid nature of the toxicity?

Segal (1972) was first to demonstrate an effect of sulfide on neuronal property or activity that was not biochemical in nature. He showed that high concentrations of sulfide inhaled by rats blocked the response of granule cells of the dentate gyrus to perforant path stimulation. McLardy (1970) observed sulfide loading into rat and guinea pig synaptic vesicles containing zinc and suggested that this loading event was responsible for the selective blockade of synaptic responses in these cells. Roth *et al.*, (1989) observed complex changes in the firing rate and action potential amplitude recorded extracellularly from crayfish sensory neuron with both sodium sulfide and H_2S. In this study, concentrations of sulfide greater than 100 μM produced irreversible effects. Warenycia *et al.* (1989b), using neuroblastoma cells, could not find any effects of high concentrations of sulfide (e.g. 50 x LD_{50}) on tetrodotoxin (TTX)-sensitive sodium channels (responsible for generation of action potentials). This study also found that sulfur containing amino acids such as taurine and cysteic acid (putative excitatory and inhibitory neurotransmitters respectively) did not alter the function of these channels. However, they both reversibly abolished the sodium currents carried by these channels in the presence of NaHS or following incubation in NaHS. Since taurine and cysteic acid are abundant in the CNS and are spontaneously released (Hudson *et al.*, 1987) the authors speculated that sulfide could interact with these compounds *in vivo* to alter neuronal function. Baldelli *et al.*, (1990), using the *in vitro* hippocampal slice preparation, showed that sulfide reversibly inhibited spontaneously active CA_1 pyramidal neurons. In this study, the observation by Warenycia *et al.* (1989b) in neuroblastoma cells (lack of effect of sulfide on the TTX-sensitive sodium channel) was corroborated since sulfide did not alter either action potential amplitude or duration in these cells. Furthermore, he found that sulfide directly inhibited synaptic transmission in this preparation and also caused a direct membrane hyperpolarization. This hyperpolarization was found to be most probably due to an increase in K^+ conductance. Upon washout of sulfide, he observed a further hyperpolarization which was sensitive to strophanthidin (an ATPase inhibitor) which he referred to as " washout hyperpolarization".

Other CNS related studies of sulfide toxicity have been conducted following low level protracted (subacute or chronic) exposures. Hannah *et al.* (1988, 1989) systematically described changes in developing mammalian CNS following low level exposure (75 ppm or less) during fetal development to 21 days postpartum. They observed an increase in cerebellar purkinje cell density, a decrease in granule/Purkinje cell ratio of developing rats. Furthermore, they reported an increase in the dendritic architecture and growth processes. Neurochemical studies on levels of putative amino acid neurotransmitters in the cerebella of these developing rats showed decreases in aspartate, GABA, glutamate, and taurine but not glycine (Hannah *et al.*, 1989).

II. THE CENTRAL NERVOUS SYSTEM AND NEUROTRANSMISSION.

The CNS is a complex organ composed of fundamental units called neurons. These are organised into anatomical regions with complex networks which may specialise in the performance or control of one or more physiological functions. Neurons within a region communicate with one another or with neurons in other regions which may be specialised for other functions. Communication between neurons is a complex but rapid process comprising both electrical and chemical components. Several processes work in concert leading to successful and precise processing and transfer of information from the source (presynaptic element) to target (postsynaptic element). These include: *a*. synthesis, transport and/or storage of neurotransmitter substances in axon terminals; *b*. release of the transmitters upon arrival of the appropriate signal; *c*. diffusion across the synapse and interaction with appropriate receptors on postsynaptic dendrites and/or soma leading to the production of postsynaptic potentials (PSP), which may be excitatory or inhibitory; *d*. the PSP would then facilitate or inhibit the generation and propagation of action potentials (APs) along the axon; *e*. degradation or reuptake of the transmitter to terminate the signal. The PSP and the APs are electrical events produced by ions passing through appropriate membrane channels.

Despite the enormous and complex neural connections of the CNS, relatively few chemical substances act as neurotransmitters in the relay of information within the CNS. Thus, one transmitter may act in several nuclei to produce similar or entirely different responses that are appropriate to the function of the nucleus.

a. Respiratory Control and Modulation by Neurotransmitters.

As earlier indicated, sulfide lethality following exposure results mainly from paralysis of central respiratory drive. The generation and maintenance of respiratory rhythmicity is from brain stem nuclei which communicate with one another and with peripheral respiratory organs by means of neurotransmitters. Some transmitter substances, not directly involved in respiratory processes, may directly or indirectly modulate these processes. The role or effects of various transmitters or putative transmitter substances, including modulators have been extensively studied. Although this role is examined in detail here, it should be noted that these substances are also involved in neurotransmission in several nuclei that show perturbation in function following sulfide intoxication e.g. dopamine and motor control in the caudate-putamen (see review by Fishman *et al.*, 1983).

Most of the studies of the effects or roles of various transmitters in respiration were *in vivo*, whereby the activity of brain stem neurons could be correlated with phrenic nerve activity (motor afferent to respiratory muscles). Hence, most studies were done by extracellular recording within putative nuclei and therefore detailed mechanisms of the effects of transmitters and their agonists is generally lacking. *In vitro* experiments are generally difficult to do and interpretation of data complex because nuclei are known to contain mixtures of cells some of which do not control or influence respiration (Segers *et al.*, 1985). Also, some respiratory nuclei are not well organised and compact for access by intracellular microelectrodes, but are diffuse isolated clusters making it more difficult record from their neurons (Vibert *et al.*, 1979).

The brain stem pontine and medulla regions are replete with clusters of cells (nuclei) implicated in generation or modulation of respiratory rhythm, either through primary afferents to other centres and to peripheral cardiopulmonary organs or via reciprocal circuitry with these nuclei and structures (Berger, *et al.*, 1977; Feldman, 1981; Feldman, *et al.*, 1976). Respiratory control is therefore a complex process involving both peripheral and central mechanisms working in concert to produce the rhythmic inspirational and expirational events that constitute respiration. Primary generators of rhythmicity originate mainly from the brain stem (pons and medulla) nuclei (Feldman, 1981; Berger *et al.*, 1977). The primary trigger of respiratory changes seems to be mainly arterial carbon dioxide and oxygen tensions (Mueller *et al.*,1982). Both peripheral and central sensors monitor these tensions to produce the appropriate response necessary to maintain normal respiration (Dempsey and Forster, 1982; Mueller *et al.*, 1982).

Three groups of neurons in the medulla have firing patterns which correlate with the respiratory cycle (Segers *et al.*, 1985; Mueller *et al.*, 1982).

1. The ventral medullary respiratory group (VRG) which are anatomically associated with the nucleus ambiguus, the nucleus retroambiguus and the retrofacial nucleus. These nuclei contain cells which discharge maximally during inspiration (I-cells), during expiration (E-cells) and phase-spanning cells (I-E or E-I cells).
2. The dorsal respiratory group (DRG) which are located in or near the nucleus tractus solitarus (NTS) and are mainly inspirational (I-cells) neurons.
3. The rostral group (RG) which also contain cells of all three types and are located in the medial and lateral parabrachial nucleus (PBN) and the Kolliker-Fuse (KF) nucleus; both of which are also described as the pneumotaxic centre. Neurons from these nuclei project to both motor nuclei in the spinal cord which innervate the diaphragm and intercostal muscles and to cells in the other respiratory cell groups (Mueller *et al.*, 1982). Furthermore, several reports of neurons, apparently more diffuse and less well organised or defined have been described throughout the reticular formation, from the brain stem to the diencephalon, whose neuronal activity modulate respiration (Vibert *et al.*, 1979). Also, respiration can be altered voluntarily or reflexly in response to changes in the mechanical properties of the thorax, during eating, coughing, exercise and changes in metabolic rate (Berger *et al.*, 1977; Wyman, 1977). For these reasons, the regulation of respiration has been difficult to define

accurately. However, the three groups of nuclei described above may be the major determinants of central respiratory drive.

b. Modulation of Respiration by Biogenic Amines.

Although none of the brain nuclei known to synthesize, store and release biogenic amines have been shown to contain cells whose firing pattern correlates with respiratory rhythmicity, most of these nuclei project afferents to, and in turn receive inputs from, one or more of the major brain stem respiratory groups. Hence, their influence on respiratory related nuclei and neurons have been extensively studied. Neuromodulators may either modify the whole respiratory cycle or selectively modify various portions of the cycle (Moss *et al.*, 1986).

i. Serotonin (5-hydroxytryptamine, 5-HT)

Serotonin-containing neurons in the brain stem are located in the raphe nuclei of the midbrain, pons and medulla (Bjorklund *et al.*, 1971; Dahlstrom and Fuxe, 1965). The largest of these nuclei is the dorsal raphe nucleus, which projects to and receives inputs from, the smaller ventral groups. Serotonin levels in brain stem nuclei have been determined (Palkovits *et al.*, 1963). Appreciable amounts were found in the PBN, locus coerulus, NTS and nucleus ambiguus. Several other nuclei, known to fire in phase with respiration, also contain significant amounts of serotonin. Thus, nuclei which contain cells that fire in synchrony with the respiratory cycle receive an appreciable amount of serotonin containing terminals (Mueller *et al.*, 1982). Also, functional activity of serotonergic neurons have been observed to be affected by CO_2 tension (Carlsson *et al.*, 1977). Florez *et al.* (1972), observed that the serotonin synthesis inhibitor, p-chlorophenylalanine caused an increase in respiratory related parameters in decerebrate cats. Armijo and Florez (1974) further showed that precursors of serotonin (L-tryptophan and 5-hydroxytryptophan) also caused respiratory depression in cats. These effects were shown to be potentiated by monoamine oxidase inhibitors (MAOI), while being prevented by amino acid decarboxylase inhibitors. Lundberg *et al.* (1980) corroborated these findings in halothane-anaesthetized rats using serotonin agonists. This group further showed that the serotonin effects on respiration were potentiated when upregulation of serotonin receptors was induced by injection of 5-7-dihydroxytryptamine. The respiratory depression was present whether the 9th and 10th cranial nerves were intact or severed, suggesting a central mechanism of action. These effects were not due to interaction with the anaesthetic as similar effects were observed using different anaesthetics (Lambert *et al.*, 1978). Also, decreases in respiratory parameters have been observed in awake rats following serotonin depletion with p-chlorophenylalanine and p-chloroamphetamine (Olson *et al.*, 1979). This is further evidence that the respiratory depressant effect of serotonin is not secondary to an interaction with the anaesthetic. Mitchell *et al.*, (1980), however observed an increase in respiratory parameters following depletion of serotonin in conscious goats. Other workers have also observed respiratory stimulant effects with serotonin (Armijo *et al.*, 1979; Millhorn *et al.*, 1980).

When serotonin is iontophoresed onto CNS neurons, it produces both an excitatory and inhibitory responses. However, only the excitatory response is antagonised by methysergide (Aghajanian and Wang, 1977). Methysergide has been observed to reverse the respiratory inhibition produced by serotonin agonists (Lundberg *et al.*, 1980; Mueller *et al.*, 1980), implying that the respiratory depressant effect of serotonin is via an excitation. Electrophysiological evidence (DeMontigny and Aghajanian, 1977; Lalley, 1982) showed that this effect of serotonin was not via presynaptic autoreceptor activation (i.e inhibition of serotonin release) but rather through activation of postsynaptic receptors at some other locus. Fallert *et al.* (1979), iontophoresed serotonin on to respiratory related medullary neurons of urethane-anaesthetized rabbits and found that excitation was the prevalent response in phase-spanning neurons (I-E cells), while most of the inspirational neurons (I-cells) responded with inhibition more often than excitation. A subpopulation of either cell type could be responsible for the respiratory effects of serotonin. To date, the exact locus of action and mechanism by which serotonin produces its respiratory effects is unclear. However, that serotonin modulates respiration has been well established, although research findings are unequivocal regarding the exact effect of serotonin. Thus, whether by inhibition or excitation, serotonin may modulate respiratory oscillations by its direct interaction with brain stem respiratory related neurons. Conversely, serotonergic neuronal activity may be altered by the activity of respiratory related neurons or events (Sole *et al.*, 1978). Serotonergic neuronal activity may also be influenced by synaptic inputs from nuclei which directly or indirectly regulate respiration. For example, the dorsal raphe nucleus receives, and its activity is greatly influenced by a noradrenergic input from the LC (Baraban and Aghajanian, 1980).

ii. Dopamine

Dopamine has been shown to be present in brain stem areas that are relevant to respiration (Lackner, 1980; Versteeg *et al.*, 1980). Peripherally, dopamine depresses chemoreceptor activity in some species (man) while facilitating it in others (cats and dogs; Black *et al.*, 1972). Apomorphine, a dopamine receptor agonist, when given intravenously (IV) or intraperitoneally (IP) to halothane-anaesthetized rats, produces an increase in respiratory rate (Lundberg *et al.*, 1979). In this same study, haloperidol, a dopamine receptor antagonist, at doses that did not affect untreated rats, antagonised the respiratory effects of apomorphine. These effects were not altered by de-afferentation of peripheral inputs but were potentiated by treatment with 6-hydroxydopamine, suggesting a central locus of action. However, other investigators observed a decrease in respiratory rate in chloralose-anaesthetized rats following iv apomorphine (Bolme, *et al.*, 1977). These differences have been attributed to the anaesthetics as differences have been observed in the release of ^{3}H-dopamine under different anaesthetic agents (Nieoullan and Dusticier, 1980). Furthermore, differences have been observed on the effect of apomorphine on respiratory rate in rats anaesthetized with different agents (Schaffer, 1958).

Dopamine effects on respiration also seem to vary with the level of activity of the CNS and the dose of agonist used (Hedner *et al.*, 1982). At lower doses, agonists seem to activate only presynaptic autoreceptors leading to decreased dopamine release and therefore a decreased response. Higher doses however, produce direct postsynaptic receptor activation and hence excitation (Carlsson, 1975). Biphasic responses have also been recorded with apomorphine in halothane-anaesthetized rats (Hedner *et al.*, 1982). This biphasic effect has been observed with dopamine itself, the initial depression being sensitive to pretreatment with MAOI while the later excitation was enhanced by MAOI (Mueller *et al.*, 1982). Using the dopamine agonist flupenthixol, Bohmer *et al.*, (1979) showed that, in urethane-anaesthetized rabbits, inspiratory neurons were excited while responsive expiratory or phase-spanning neurons were inhibited. The direct effect of dopamine on respiration could therefore be both excitatory and inhibitory depending on several factors. In addition to these direct effects, dopamine also interacts with other transmitter systems to indirectly affect respiration. For example, Grabowska (1974), observed that apomorphine accelerated the disappearance of serotonin after inhibition of its synthesis. Also, dopamine neuronal activity may alter noradrenergic neuronal function. Apomorphine has been reported to increase noradrenaline turnover in several animal species (Perssons and Waldeck, 1970) and brain areas (Maj *et al.*, 1977).

iii. Noradrenaline and adrenaline

Noradrenaline is produced and stored in three distinct nuclei in the brain: the locus coeruleus (LC), the subcoeruleus and the dorsal motor nucleus of the vagus. The pontine LC appears to be the main source of adrenergic innervation to the CNS. It is also located close to, and may actually be part of, the pneumotaxic centre of the parabrachial nucleus (PBN; Dahlstrom and Fuxe, 1964). Axonal transport studies and immunocytochemical staining have shown that noradrenergic terminals arising from the LC terminate in the dorsal motor nucleus, the nucleus ambiguus and the NTS (Westlund and Coulter, 1980). It was further shown that all brain stem nuclei receive noradrenergic inputs from either the LC or the subcoeruleus or both. Also, small clusters of cells with positive response to antisera to phenylethanolamine-N-methyl transferase (a marker of adrenergic neurons) have been located in the brain stem. These cells innervate the spinal cord, the LC and other nuclei (Howe *et al.*, 1980). Therefore, the noradrenergic and adrenergic nuclei, like the serotonergic nuclei are also closely located to and interact extensively with neurons that fire in phase with respiration.

Whelan and Young (1953) first reported that peripheral administration of adrenaline stimulated respiration. Several studies on the effect of noradrenaline and adrenaline on respiration have yielded conflicting results. Padron and Florez (1978), while investigating the mechanism of bromocriptine-induced cardiovascular and respiratory effects, observed that part of the bromocriptine-induced increase in respiration was blocked by phentolamine, indicating, based on the selectivity of phentolamine for α adrenoceptors, that the latter receptors were involved in respiratory control. Also, the amphetamine-induced increase in respiratory rate in decerebrate rats was shown to be blocked by phentolamine but not propranolol (Mediavilla *et al.*, 1979), further evidence for α, but not β, adrenoceptor mediation of this effect. While several workers have observed respiratory stimulation by adrenaline, others have found decreases in respiration following administration of selective agonists. For example intravenous administration of the α_2 agonist, clonidine was found to decrease respiration (Bolme *et al.*, 1973). This appeared to be caused by the selective activation of α_2 adrenoceptors as α_2 an selective antagonist (yohimbine) abolished this effect of clonidine (Bolme *et al.*, 1974). Further to these, other investigators found no effect on respiration when both phentolamine and propranolol, at doses that altered cardiovascular responses, were given to awake goats challenged with α and β adrenoceptor agonists (Weinberger *et al.*, 1982).

During *in vivo* microiontophoretic studies, application of isoproterenol, a β agonist, to respiratory related neurons in urethane-anaesthetized rabbits produced excitation of inspiratory cells while noradrenaline produced both excitation and inhibition in these neurons, although excitation was more common. Using a similar preparation and identical techniques, Bohmer *et al.* (1979) observed inhibition of expiratory cells, but no effect on inspirational or phase spanning neurons in response to yohimbine, a selective α_2-adrenoceptor antagonist. Clonidine may therefore depress respiration through activation of α_2 adrenoceptors on expiratory neurons. In a comparative study, Champagnat *et al.* (1979) found that adrenaline, a more specific β-adrenoceptor endogenous transmitter, depressed respiratory-related neurons of decerebrate cats more than noradrenaline did, suggesting a β-adrenoceptor mediation of the respiratory depressant action of noradrenaline and adrenaline.

Although literature on the effect of noradrenaline and adrenaline on respiration are conflicting, the general trend seems to be that activation of α-

adrenoceptors produces respiratory stimulation while β- adrenoceptor activation causes inhibition of respiration. The effect of adrenaline and noradrenaline may be further complicated by the fact that there seems to be a reciprocal interaction between the noradrenergic and the serotonergic systems. For example, electrolytic lesions of the raphe nuclei, in cats, increased noradrenaline turnover in the cerebellum and cortex (Pujol, 1973). Also, chemical depletion of serotonin by 5,6-dihydroxytryptamine results in increases in tyrosine hydroxylase and β-dopamine hydroxylase activity in the LC (Keane *et al.*, 1978). Furthermore, biochemical and histochemical data show noradrenergic afferent fibres from LC to the raphe nuclei (Aghajanian & Wang, 1979; Roizen & Jacobowitz, 1976). Serotonergic neurons in turn send afferents to the LC from the raphe nuclei (Leger *et al.*, 1980). Thus, these systems may modulate the effect of each other on respiratory neuron activity.

c. Modulation of Respiration by Neuroactive Amino Acids.

i. γ- amino butyric acid (GABA)

This is an abundant amino acid in the CNS. Its amount varies from region to region with high concentrations in brain stem nuclei and spinal cord (Fahn, 1979) while the respiratory relevant nuclei (PBN, NTS, LC) contain moderate amounts (Van der Heyden *et al.*, 1979). The role of GABA as a central inhibitory neurotransmitter is well established (Enna & Maggi, 1979; Krnjevic, 1974). In general, GABAergic neurons are intrinsic neuron (interneurons) which are activated by recurrent colaterals or feed forward inputs to mediate inhibitory responses.

Peripheral iv injection of GABA has yielded both respiratory stimulatory and inhibitory effects or both. These results could be due to differential CNS penetration as GABA passes the blood-brain barrier poorly (Purpura *et al.*, 1958). However, intraventricular injection in anaesthetized rats produce a dose-dependent, rapid and marked depression of respiration (Hedner *et al.*, 1981). This depressant effect has also been observed in cats (Yamada *et al.*, 1981). The $GABA_A$ selective agonist, muscimol, mimicked the effect of GABA, although less potently (Hedner *et al.*,1981). This difference in potency may mean GABA produces its effect by both $GABA_A$ and $GABA_B$ receptor activation. The $GABA_A$ receptor antagonist, bicuculline and the chloride channel blocker picrotoxin both possess respiratory stimulatory effects (Hedner *et al.*, 1981; Hirsh & Wang, 1975). These antagonists, when applied by iontophoresis, induce discharge activity in inspiratory neurons in the expiratory phase only, without altering the timing of the cycle. This suggests that GABA acts to tonically depress a population of respiratory related neurons (inspiratory neurons; Denavit-Saubie *et al.*, 1982 & 1983; Moss *et al.*, 1985). Iontophoretic application of GABA to respiratory related neurons in the pneumotaxic centre and medulla of cats resulted in greater depression of expiratory than inspiratory neurons. However, in the NTS, nucleus ambiguus, and some pneumotaxic centre neurons, inspiratory neurons were selectively depressed by GABA (Denavit-Saubie *et al.*, 1981). Also, blockade of GABA uptake with nipecotic acid potentiated the respiratory depressant effect of subsequently administered GABA.

GABA, by its direct action may regulate the firing pattern of respiratory related neurons. In addition to these direct effects, GABA may also influence respiration by its effect on other neuronal systems which directly affect respiration. For example GABA is known to influence the activity of catecholamine and serotonin containing neurons (Carlsson *et al.*, 1977).

ii. Glycine

This is another amino acid that has been shown to possess neuronal depressant effects. Respiratory depression has been observed following intracisternal and intraventricular administration in anaesthetized rats (Sgaragli & Pavan, 1972; Hedner *et al.*, 1982). Electrophysiological studies in brain stem nuclei have shown glycine to produce a hyperpolarization of neurons which is sensitive to blockade by strychnine (Hösli *et al.*, 1969). Iontophoretic application of strychnine to respiratory neurons caused an increase in spike discharge of inspiratory neurons only at end-inspiration, thus delaying the onset of expiration (Denavit-Saubie *et al.*, 1982 & 1983). Since strychnine is an antagonist of all glycine-like amino acid transmitters (alanine, taurine), this observation may mean that a glycine-like neurotransmitter participates in the off-switch mechanism that terminates inspiration abruptly and permits the onset of expiration (Moss et el., 1985). Toleikis *et al.* (1979) in a comparative study observed that glycine was less effective than GABA in the depression of phasic activity of respiratory neurons. Glycine, like GABA, may act directly to modulate respiratory neurons or indirectly via its effect on other nuclei that influence respiration.

iii. Alanine

This amino acid is present in low amounts in mammalian CNS (Perry *et al.*, 1971) but has potent depressant effects on central neurons (Curtis *et al.*, 1959). Intracerebroventricular (ICV) injection of alanine into anaesthetized rats, in a dose comparable to glycine and GABA, induces respiratory depression (Hedner *et al.*, 1982). This effect was probably elicited within the brainstem as it has been shown to suppress the spontaneous firing of brain stem neurons. Also, the effects of alanine were similar to those of glycine and both were antagonised by strychnine (Hösli *et al.*, 1969). Thus the effect of alanine on respiration may be via activation of a glycine receptor.

iv. Taurine

This amino acid is abundant in the CNS and has a heterogenous distribution in the brain (Barbeau *et al.*, 1976). Iontophoretic application of taurine results in inhibition of spontaneous firing of neuron from several brain regions including the brain stem (Curtis *et al.*, 1971). Because its effects are antagonised by strychnine, taurine, like alanine, has been said to have a glycine-like effect (Curtis *et al.*,1971b). However, ICV administration into rats produces a greater respiratory depression compared to glycine and alanine (Hedner *et al.*, 1982).

v. Glutamate and Aspartate.

L-glutamate has been demonstrated to be the primary excitatory neurotransmitter in several areas of the brain (reviewed by Watkins and Evans, 1981) including the NTS (Talman *et al.*, 1980) where it produces changes in respiration among other responses. Iontophoretic application of glutamate and aspartate onto respiratory related neurons produced identical responses with equal potency (Toleikis *et al.*, 1979). These substances increase the activity of both inspiratory and expiratory neurons. McCrimmon *et al.* (1986), showed that glutamate and its selective agonist, N-methyl-D-aspartate (NMDA), produced excitation of respiratory related neurons in cat brain stem which was sensitive to blockade by selective antagonists. This indicated a specific action on receptors rather than an indirect, non-specific action. Foutz *et al.* (1988), observed excitation of medullary respiratory neurons with NMDA and quisqualate, the NMDA effect being blocked by DL-2-amino-7-phosphonoheptanoic acid (AP7), an NMDA receptor selective antagonist. Application of AP7 alone decreased the firing rate of these neurons. Other selective, non-competitive glutamate receptor antagonists, such as MK-801, phencyclidine and ketamine, upon systemic administration, blocked the effects of NMDA while inducing an increase in duration of inspiratory neuronal activity, with no change in expiratory neuronal discharge. This led to the hypothesis that glutamate may contribute to the generation of respiratory rhythm by influencing the switching off of inspiratory phase of respiration. Glutamate may therefore play a crucial role in the generation and/or maintenance of respiratory rhythm.

III. STATEMENT OF PURPOSE

The main aim of this study was to explore the basic neural alterations and the mechanisms that underlie the various clinical symptoms that are seen following acute exposure to H_2S, in order to facilitate the rational development or choice of potential antidotes to acute toxic sulfide poisoning. Any alterations that are seen on CNS function must occur rapidly enough to account for the quick occurrence of toxic manifestations. More importantly, because death from lethal exposure occurs via inhibition of central respiratory drive (Amman, 1986), the neuronal properties of nuclei and neurons that control or influence respiration will be examined in more detail. These studies will therefore test the hypothesis that hydrogen sulfide toxicity is via alteration in neural mechanisms in the CNS. Furthermore information from these studies will aid in the rational choice and testing of potential antidotes to acute lethal exposure to sulfide.

Alteration in the structure and/or function of any of the events comprising neuronal interaction can lead to inappropriate neurotransmission and therefore cause the neurological symptoms observed during or following acute exposure to H_2S, particularly respiratory failure. These studies will examine the effects of sulfide (NaHS) on basic neuronal constituents, properties and function. In this regard, the following hypotheses will be tested: *a*. that sulfide alters transmitter turnover (synthesis and storage); *b*. that sulfide alters the release and probably the fate of transmitters following release; *c*. that sulfide will modulate synaptic transmission, resting membrane properties and agonist-induced responses in neurons. Both *in vivo* and *in vitro* experimental techniques will be employed to investigate these chemical and electrical events.

Five brain regions were selected for the study because of their known involvement in the various neurological symptoms observed during or following poisoning. The cerebral cortex for its control of sensory perception and memory (Scoville & Milner, 1957; Penfield & Milner, 1958; Milner, 1970; Iverson & Weiskrantz, 1970); the hippocampus for its role in memory (Scoville & Milner, 1957; Penfield & Milner, 1958; Mishkin, 1954; Iverson & Weiskrantz, 1970); the striatum (caudate-putamen) and cerebellum for their involvement in motor control (Ito, 1984; Delong & Georgopoulos, 1981; Aldridge, *et al.*, 1980; Kimura, 1987; McGeer & McGeer, 1987); and finally, the brainstem for containing most of the centres that control vital processes such as cardiovascular function and respiration (Lunsden, 1922; Bianchi & St. John, 1980; Bianchi & Barillot, 1982; Segers, *et al.*, 1985). The studies in this project, will be both biochemical (neurochemistry) and electrophysiological in nature. The chemical substances that have been found to carry out neurotransmitter function (meet the stringent requirements of neurotransmitters) in the CNS are acetylcholine, noradrenaline, adrenaline, dopamine, 5-hydroxytryptamine, GABA and glutamate (Watkins and Evans, 1981). The effect of sulfide on the synthesis, storage, release, and postsynaptic responses produced by these substances will be examined. The above studies will also look at putative amino acid neurotransmitter candidates such as alanine, glycine and taurine.

Toxicological studies on potential antidotal substances will be conducted in rats to establish the safety of these substances and their ability to reverse or protect against sulfide toxicity will also be examined.

CHAPTER II

MATERIALS AND METHODS

I. NEUROCHEMICAL STUDIES

Experimental Animals

Male Sprague-Dawley albino rats (*Rattus norvegicus*) weighing between 200-350 g were obtained from the University of Alberta breeding program (Biological Sciences). 2-3 animals were housed per cage with sawdust floors and kept on a 12 hour light-dark cycle at a constant ambient temperature of 24°C. Rat chow ("Wayne rodent blox") and water were made accessible to the rats *ad libitum*.

A. EX VIVO EXPERIMENTS: *Total Transmitter Synthesis and Storage Studies.*

a. Dissection

Male Sprague-Dawley rats (*Rattus norvegicus*) weighing between 200-350 g were used in these studies. Fresh solutions of sulfide were made by dissolving NaHS (Aldrich Chemical Co., Milwaukee, WI) in saline. Appropriate volumes were then injected intraperitoneally (IP), making sure no animal received injection volumes greater than 10% of its body weight. Doses corresponding to 10 and 30 mg/kg were used. Control animals received equivalent volumes of saline. Animals not succumbing to sulfide were killed by a blow to the head followed by cervical dislocation. The mean time to death was approximately 2 min subsequent to IP administration of NaHS, so 2 min following injection of sulfide, live animals were considered as having not succumbed to sulfide. Brains were quickly excised and dissected on ice into cerebellum, brainstem (medulla and pons), hippocampus, striatum and cerebral cortex. The hippocampus was taken from regions corresponding to 2.3-4.3 mm posterior to bregma and lying beneath the cerebral cortex. The striatum was taken from the region corresponding to 2.2 mm anterior to bregma to 0.2 mm posterior to bregma (anterior striatum). Cerebral cortical samples were taken from the region overlying the striatum and the brainstem samples corresponded to the region 2.5 mm anterior to the interaural line (IAL) to 4 mm posterior to the IAL. All co-ordinates according to the atlas of Paxinos and Watson (1986).

b. Tissue Processing

i. Amino Acids

Immediately upon dissecting out each region, it was flash frozen in 2-methyl butane cooled by liquid nitrogen and stored at -30°C until processing (within 2-7 days). Pooled brain regions were weighed and homogenised in 1 ml of 0.1 M HCl using a sonicator or teflon pestle. Homogenates were then immersed in a 100°C water bath for 5 min to inactivate enzymes and subsequently centrifuged at 14,000 x g for 15 min. Supernatants were then filtered through a 0.22 μm "GS Millipore" filter paper and transferred to sample vials and frozen at -80°C until analysis by HPLC (see below) within 7 days.

ii. Catecholamines.

The dissection procedure here was identical with that in the amino acid studies. However, homogenization was done by sonication in 4-10 volumes of ice cold 0.4 N perchloric acid ($HClO_4$). Centrifugation was done at 11,800 x g for 20 min.

c. Data Analysis

Amino acid levels in picomoles were transformed into amount per tissue wet weight using a statistics program written for this purpose. In doing this calculation, the original weight of the tissue was considered in addition to any dilutions (dilution factor) that were made prior to HPLC analysis. Various amino acids from various brain regions were then compared using the Student-Newman-Keul's multiple comparison procedure (Dowdy and Weardon, 1983). Catecholamine levels in the various brain regions were compared by analysis of variance and Duncan's Multiple-Range Test (Dowdy and Weardon, 1983); $p < 0.05$ was considered significant.

B. IN VIVO EXPERIMENTS: *Release Studies.*

a. Anaesthesia

Rats were anaesthetized by intraperitoneal (IP) injection of urethane (ethyl carbamate) at a dose of 1.2-1.5 g/kg, starting with the lower dose and then giving supplemental doses of 0.1 g/kg until a stable surgical anaesthesia was attained but not exceeding 1.5 g/kg of total anaesthetic. Once a surgical anaesthesia was attained, it lasted long enough that supplemental doses were no longer necessary. Since animals are incapable of maintaining proper body temperature during anaesthesia, a rectal temperature probe and a heating blanket, powered by a 12V battery connected to a servo temperature control was used to maintain body temperature at 37± 0.5°C.

b. Surgical Procedures

Once anaesthesia was attained, ear bars were put in place by sliding them into the external auditory meatus and gently pressing them until both ear drums were perforated. The ear bars were then clamped in place and the animal was then carefully lifted with the ear bars secured in place and placed in a Narashige (Model SR5; Tokyo, Japan) stereotaxic apparatus. The rat was then centred by adjusting the ear bars to equal distances on either side. A scalpel was used to expose the skull by cutting along the midline and the skin and temporal muscle were reflected laterally with a bone raspatory. Some scalp muscle was then crushed to promote clot formation. A belt driven, motorized, dentist drill was used to drill holes of 1-2 mm in diameter at previously marked locations. After the *dura* was exposed, it was cut away by first piercing it with a sharp needle and then gently lacerating it in all directions. If excessive bleeding occurred during or following this process, bone wax was used to seal up the wound and also to aid clot formation.

c. Stereotaxic Methods

To ensure accurate placement of electrodes and cannulae, a marker (usually the tip of the perfusion cannula clamped in the holder) was aligned midway between the ear bars adjusted to equal distances on either side. This position was taken as zero and medial/lateral and anterior/posterior readings of the micromanipulators recorded. The animal was then placed in the stereotaxic apparatus and centred by adjusting the ear bars to equal distances on both sides of the animal. The incisor bar was then inserted underneath the incisors and the spring loaded clamp lowered gently and secured over the snout. The incisor bar was then lowered to 3.3 mm below the interaural line (Paxinos and Watson, 1986). The marker was then lowered and if correctly positioned, touched the lambda(λ) reference point (approximately 0.4 mm posterior to the junction of the lambdoid sutures). All measurements were made with reference to lambda. In cases where the initial marker positioning was not accurate, bregma was used as the reference point since it is easier to locate (intersection of medial suture and the sagittal suture) than λ.

The hippocampal pyramidal cells in area CA1 were located at 5.2 mm anterior to λ or 3.8 mm posterior to bregma, 1.5-1.8 mm lateral from the midline, and at a depth of 2.4-2.6 mm from the surface of the brain at the point of entry. The commissural pathway (fimbriae fornix) was accessed by angular entry to a point previously determined by a marker positioned at 6.2 mm anterior to λ, 0.08-0.12 mm lateral from the midline, and 7 mm dorsal from the interaural line. The caudate-putamen (striatum) was obtained at 9.2 mm anterior to λ or 0.2 mm anterior to bregma, 2.8-3.0 mm lateral from the midline, and 4.5 mm ventral from the surface of the brain. Finally, the brainstem reticular nucleus immediately bordering the nucleus ambiguus in the dorso-ventral medullary area was obtained at 4.8 mm posterior from λ, 2 mm lateral from λ, and 2.5 mm dorsal from the interaural line.

d. Push-Pull Perfusion

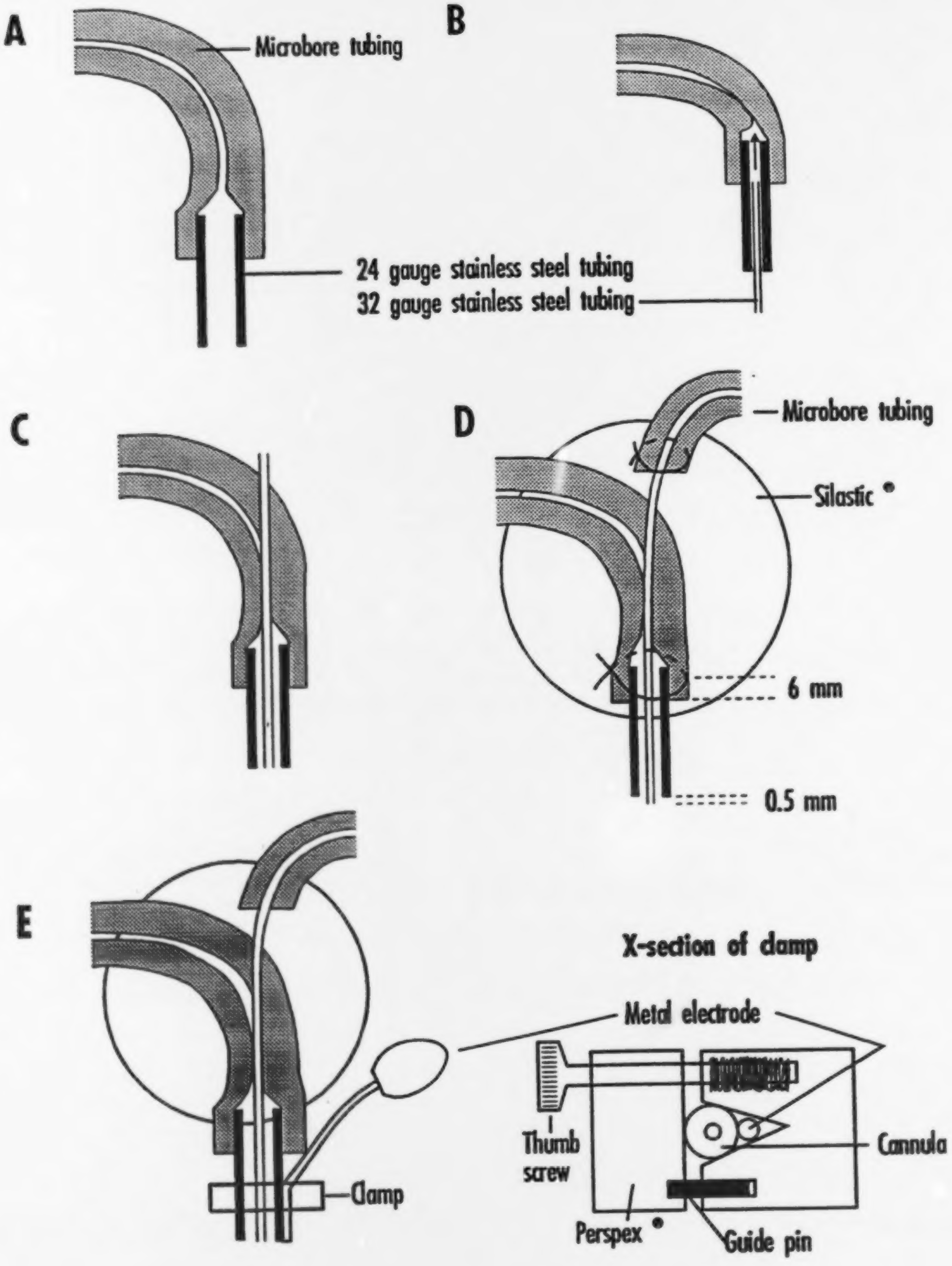
i. Construction of the Push-Pull Cannulae (PPC).

The construction of the PPC was by the method of Hudson *et al.*, (1988). Briefly, a 24 gauge, 52 mm long (outer) and 32 gauge, 62 mm long (inner) stainless steel tubing, finished to be blunt, were obtained from Popper & Sons (New Hyde Park, N.Y). Tygon® (R-3603 plastic) micro-bore tubing with internal diameter of 0.19 mm (Cole-Palmer: TV-7626-72) was stretched over one end of the outer stainless steel tubing. The inner tubing was then slowly pushed through the free end of the outer tubing until it perforated the Tygon® tubing. The inner barrel was then adjusted so it protruded approximately 0.5 mm beyond the tip of the free end of the outer barrel. The end of the inner tubing protruding from the Tygon® tubing was then fitted with another piece of Tygon® tubing and bent approximately 90° to the outer tubing. All the metal-tygon® tubing connections were over-tied with surgical thread and junctions held together with "five minute" epoxy glue. The entire T-junction was then covered with Dow-corning silastic® and cured over-night at room temperature (Fig. 1, adapted from Hudson *et al.*, 1988).

ii. Stimulation and Recording.

During perfusion of the area CA1 of the hippocampus, a bipolar tungsten electrode was placed in the fimbria fornix/dorsal hippocampal commissure permitting both antidromic and orthodromic stimulation of commissural fibres leading to enhanced transmitter release. Stimuli of between 5-10V, of 1 ms duration were

FIGURE 1. Steps in the construction and use of push-pull cannulae. A. A microbore flexible tubing (ID= 0.19 mm) is stretched over a 24-gauge stainless-steel outer barrel. B. The plastic tubing is bent sharply, and the inner barrel is pushed through the side wall of the plastic tubing. C. The inner barrel is pushed through tubing to adjust the protrusion of the inner barrel from the outer barrel. D. The inner barrel is fitted with the same diameter flexible tubing and tied with thread and the entire junction is covered with Silastic® and cured at room temperature. E. Final assembly of perfusion and recording units (recording metal electrode alongside the cannula). The clamp has a suitably angled V-cut to permit the cannula and the electrode to be clamped together tightly (Adapted from Hudson et al., 1986).

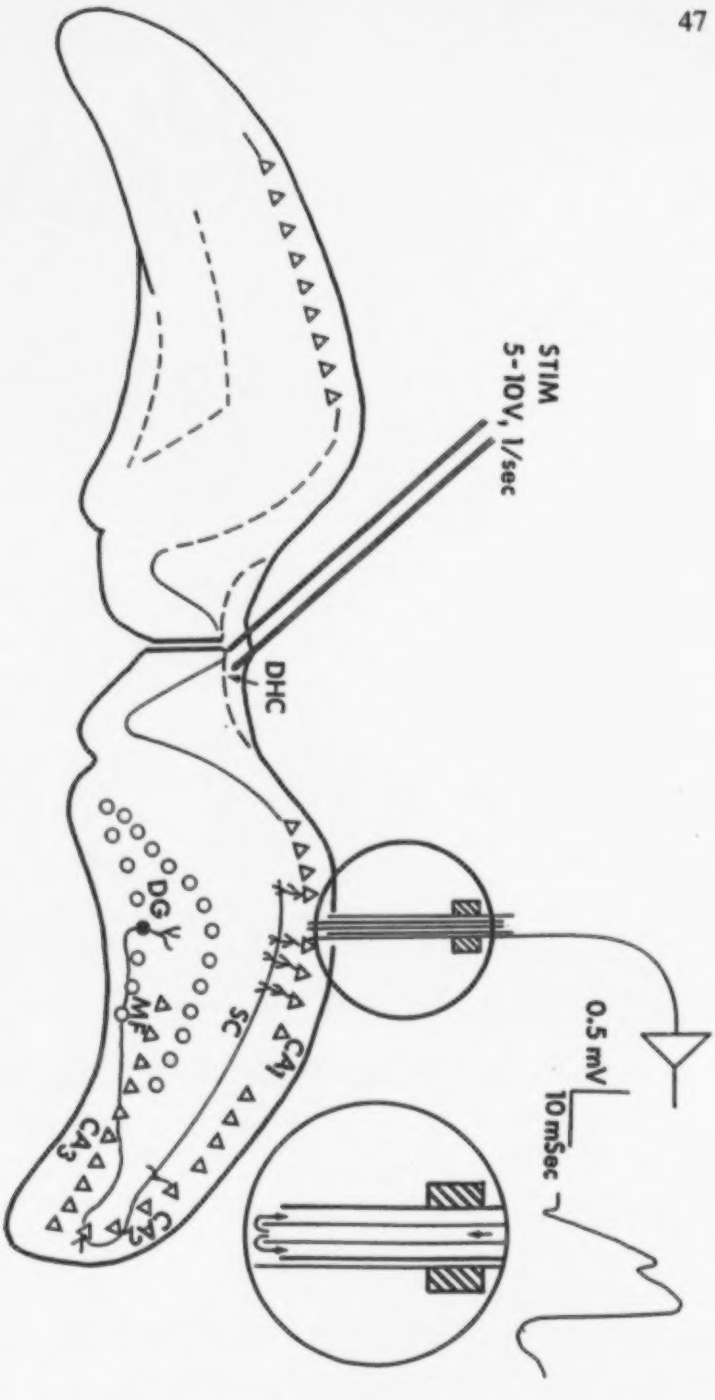

A
Microbore tubing
B
24 gauge stainless steel tubing
32 gauge stainless steel tubing
C
D
Microbore tubing
Silastic ®
6 mm
0.5 mm
E
Clamp
X-section of clamp
Metal electrode
Thumb screw
Cannula
Perspex ®
Guide pin

delivered once a second throughout the entire experiment. A unipolar, metal recording electrode (impedance, 2-5 MΩ) was screwed tightly into the same groove with the push-pull canula (ppc) and its tip accurately matched with the tip of the inner barrel of the canula (Fig. 2).

iii. Perfusion

The push pull canula was screwed tightly into the same groove (etched out on a perspex block) with the recording electrode. The above setup was then attached to a micromanipulator head stage. The canula was then zeroed to the interaural line and adjusted to the appropriate co-ordinates for the particular region as described in the stereotaxic methods. The tubing connecting the two barrels (see manufacture of cannulae) were then attached to the appropriate leads on a peristaltic pump (Peristaltic Pump P-3, Pharmacia Fine Chemicals, Sweden), the lead attached to the inner barrel being connected to the push lead on the pump while the lead attached to the outer barrel was connected to the pull lead of the pump. The other end of the push lead was then placed in a beaker containing artificial cerebrospinal fluid (ACSF, composition in mM: $NaCl$, 138; KCl, 3.40; $MgCl_2$, 0.54; $CaCl_2$, 0.95; NaH_2PO_4, 0.50; urea, 2.13; $NaHCO_3$, 2.9O; and D-glucose, 3.40). The pump speed was fixed and the pressure on the peristaltic tubing adjusted such that a total volume of 20 μl/min of perfusate was collected at the free end of the pull tubing. At the commencement of every perfusion experiment, the lines were first cleared of gases and the pump made to pump ACSF for at least 1 hour to stabilize. Although Yaksh and Yamamura

FIGURE 2. A schematic diagram of the hippocampal formation showing the placement of the stimulating bipolar electrode in the dorsal hippocampal commissure, to activate both hippocampi orthodromically and antidromically. Also shown is the placement of an extracellular metal electrode, attached to a concentric barrel push-pull cannula, the former to record the compound field potential and the population spike and the latter to retrieve released transmitters in the CA1 area. On the right is an expansion of the cannula-electrode arrangement, showing the push and pull direction of the cannula. Also displayed is a typical population spike superimposed on the compound field potential captured on a dual beam oscilloscope by a polaroid camera. DG: dentate gyrus; DHC: dorsal hippocampal commissure; MF: mossy fibre; SC: Schaffer colateral; CA: Cornus Ammonis.


STIM
5-10V, 1/sec
DHC
DG
MF
SC
CA1
CA3
CA3
0.5 mV
10 mSec

(1974) have shown this technique to closely reflect on-going changes in the local concentrations of brain substances, the recovery, quantification of neurotransmitters and interpretation of the data are complicated by inherent limitations of the technique. The sensitivity of the technique is influenced by several factors that have to be maximized or minimized to obtain optimal results. Because only minute quantities of neurotransmitters are released at a time, relatively large volumes (≥ 100 µl have to be collected to obtain amounts within the detection capability of any of the detectors used. Also, both spontaneous and evoked transmitter release can very greatly from animal to animal especially under anaesthesia. Furthermore, the reproducibility of the recovery process is dependent on the collection efficiency (the ratio of the amount of material collected by the system to the amount available for collection; Yaksh and Yamamura, 1974). This collection efficiency is dependent on the amount of protrusion of the inner barrel from the outer one and the maintenance of a constant, uninterrupted perfusion. Alteration in any of the above parameters therefore required the re-establishment of a new baseline. Inherent metabolic processes such as degradatory and reuptake processes also decrease the collection efficiency and may have to be inhibited or activated to enhance the latter. Finally, Yaksh and Yamamura (1974) observed that tissue damage (as indicated by the amount of protein in the perfusate) was minimal in isotonic solution and declined to a negligible level in 15-30 min after the commencement of perfusion. Because of these numerous sources of variability, effects were made to maximize the positive factors while minimizing the negative influences, starting from the fabrication of the ppc to the choice of appropriate analytical techniques. At the beginning of each experiment, the cannula was stereotaxically placed in the predetermined site and the animal was perfused for 30 min (being careful to ensure that blood was not drawn to clog the fine bore of the inner barrel). After this, 10 min (200 μl) samples were then collected in the following order; 2 or 3 control samples, application of NaHS (by IP injection or perfusion) and collection of 4-5 samples. For application of sulfide by perfusion, an aliquot of freshly prepared stock of NaHS was diluted to the final concentration in ACSF. The collection efficiency of GABA, TAU and probably GLY were enhanced by the inclusion of nipecotic acid (5 μM), an uptake blocker.

iv. Sample Processing.

The 10 min (200 μl) samples were collected into 500 μl microcentrifuge tubes. They were immediately centrifuged at 2800 x g for 15 min. 180 μl aliquot was then carefully pipetted into a 250 μl HPLC sample tube and 20 μl of internal standard, containing 100 μM each of cysteic acid and norvaline, was then added to produce a final volume of 200 μl. The tubes were then vortexed to produce thorough mixing and analyzed by High Performance Liquid Chromatography (HPLC) or frozen at -30° C prior to analyses within 7 days.

v. Data Analysis

For these studies, the absolute amounts of amino acids in the two samples collected before drug application were compared by the paired Student t-test to ensure they did not vary greatly (samples with $p < 0.05$ were rejected). These two samples were then averaged to give a control amount against which all other samples from that experiment, including themselves, were standardized. The standardized values of each sample in control rats (which received equivalent volumes of saline) were then compared with corresponding time-matched samples in treated rats (intraperitoneal injection or perfusion with solution of NaHS dissolved in ACSF), using unpaired t-test. $p < 0.05$ was considered as significant.

e. Verification of Site of Perfusion

Accurate placement of the tip of the PPC in the anatomical region of interest was important in the success of the experiments. Only results from animals with accurate canula placement were analyzed. Accurate placement was ensured by carefully reading the stereotaxic co-ordinates as indicated by the atlas of Paxinos and Watson. Also, only animals in the weight range recommended by the atlas were used. In the case of the hippocampus, which has a well characterized cytoarchitecture and neural pathways, accurate placement of the ppc was further ensured by adjusting the depth of the canula (with the recording electrode attached) so that the amplitude of the population spike (PS) of the pyramidal cells of area CA1, evoked by stimulation of the hippocampal commissure, was maximized. A Tektronix polaroid C30A camera was used to photograph single PS from the oscilloscope (Type 502A Dual-Beam Oscilloscope, Tektronix Inc., Portland, OR). For the other regions with less well defined neural networks, this could not be done.

At the end of each experiment, the animal was perfused intracardially, first with normal saline and then with buffered 10% formaldehyde. The fixed brain was then excised and stored in buffered formaldehyde for sectioning later on. Prior to sectioning, the fixed brain was trimmed around the site of entry, mounted on tragacanth gum and covered with O.C.T compound* (embedding medium; Miles Inc., Elkhart, IN). The sample was then placed in 2-methylbutane(isopentane), cooled by liquid nitrogen for 15 sec resulting in a solid frozen block of tissue. The block was then mounted in a cold microtome (IEC cryostat; International Equipment Company, Needham Height, MA) and sectioned. 20 μm coronal sections were cut until the path of damage from the surface of the brain to the appropriate region could be clearly seen.

FIGURE 3. A schematic diagram of the experimental arrangement of equipment used for high performance liquid chromatographic (HPLC) analysis. (WISP = Waters Intelligent Sample Processor; detector = fluorescence or electrochemical).

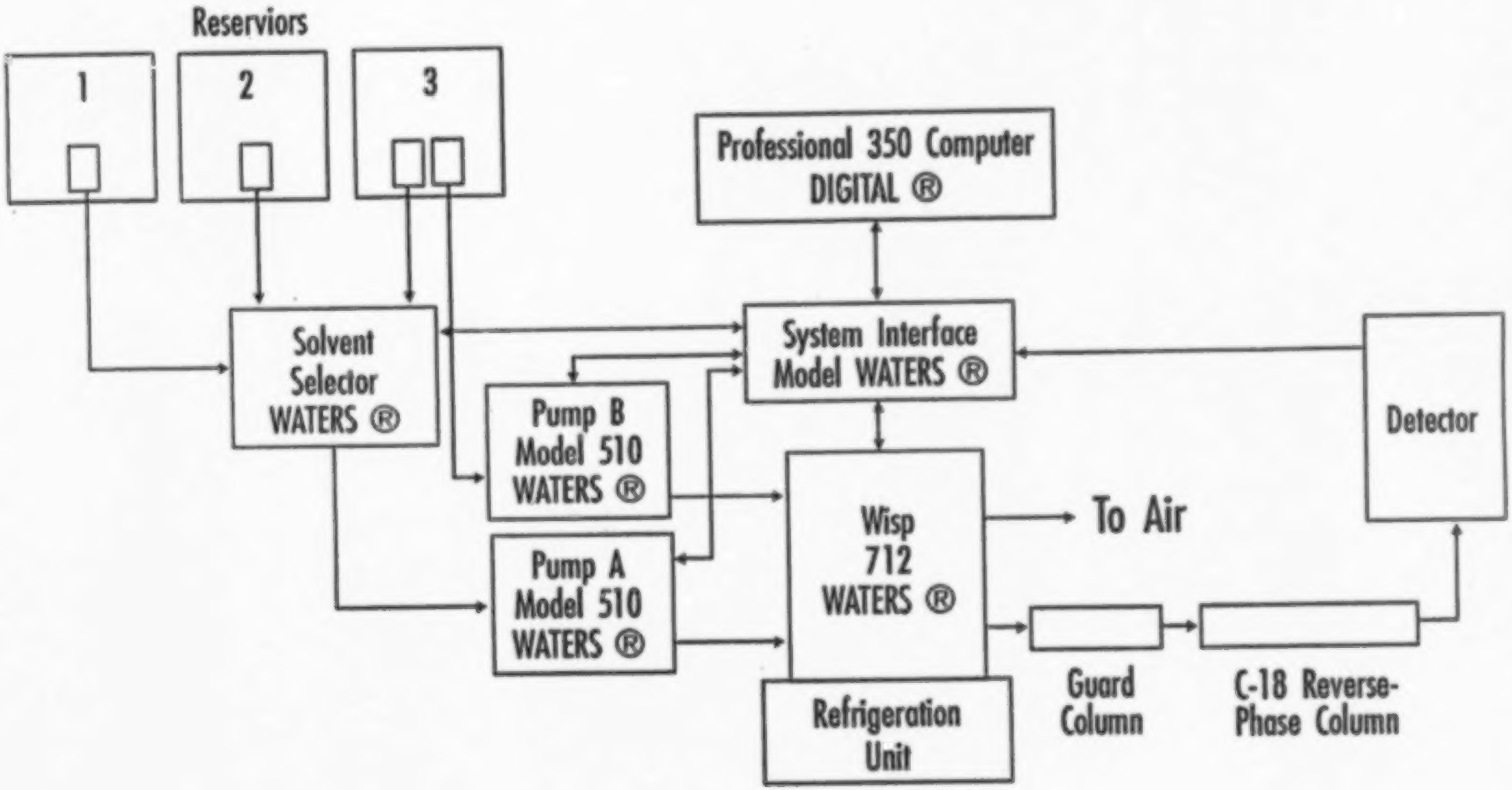
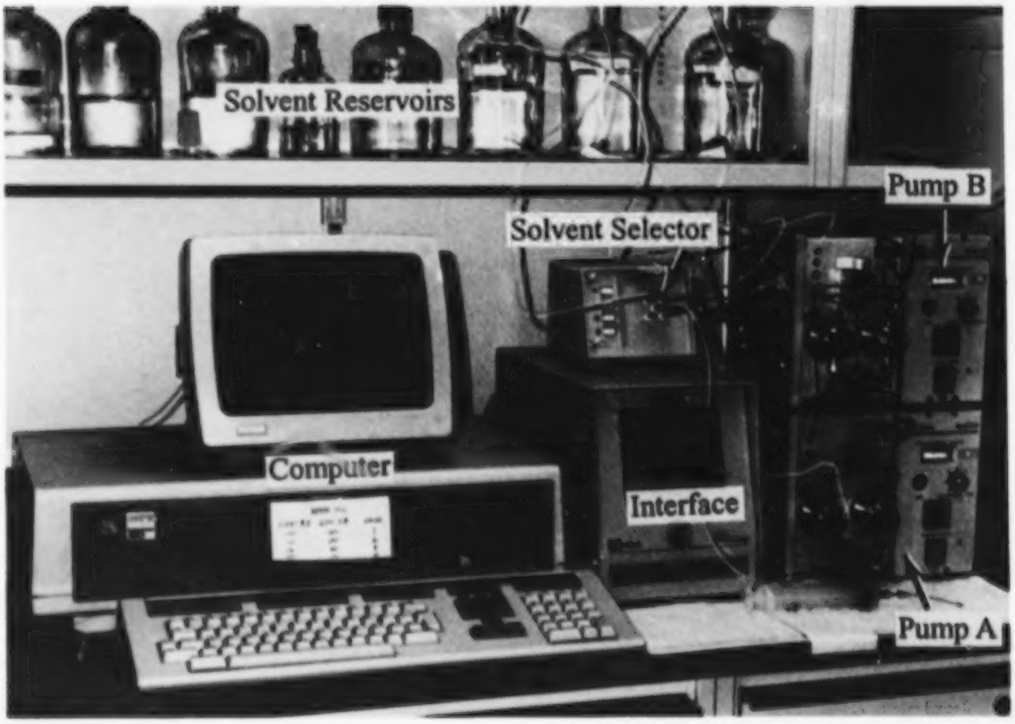
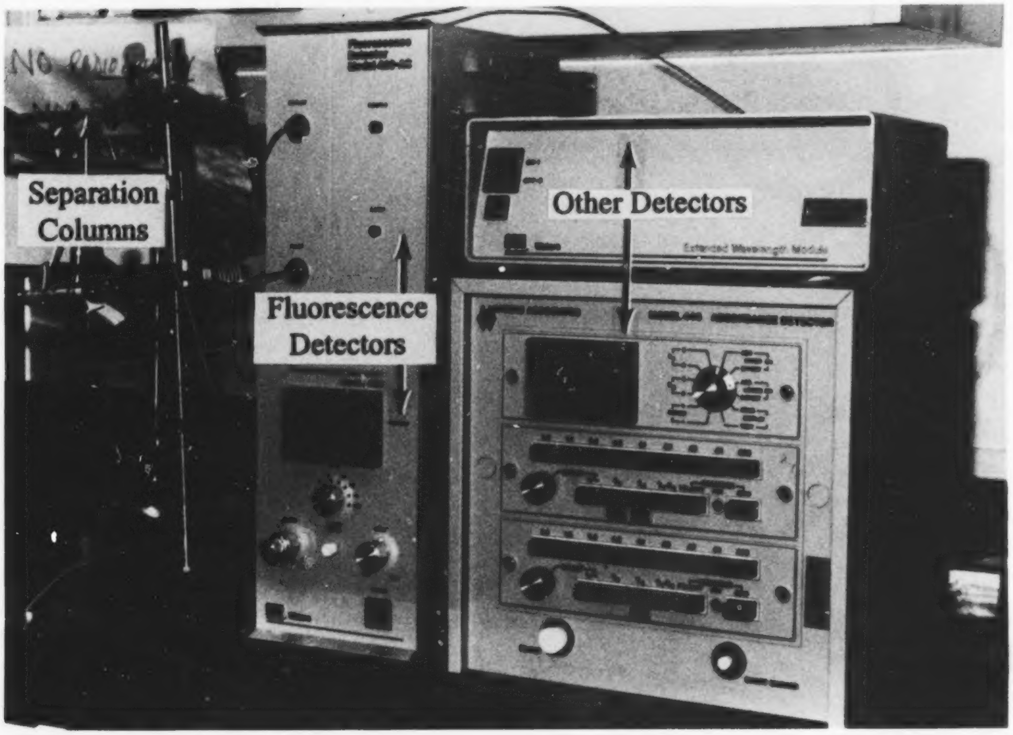

Reserviors
1
2
3
Solvent Selector WATERS ®
Professional 350 Computer DIGITAL ®
System Interface Model WATERS ®
Pump B Model 510 WATERS ®
Pump A Model 510 WATERS ®
Wisp 712 WATERS ®
Refrigeration Unit
To Air
Guard Column
C-18 Reverse-Phase Column
Detector

PLATE 1


Solvent Reservoirs
Pump B
Solvent Selector
Computer
Interface
Pump A

Plates 1 & 2 Experimental equipment for HPLC analyses.

PLATE 2



Separation Columns
Fluorescence Detectors
Other Detectors
Extended Wavelength Module

f. High Performance Liquid Chromatography (HPLC)

i. Instrumentation and Preparation Prior to Analysis.

The HPLC system employed in all these studies comprised a Waters 712 WISP (Waters Intelligent Sample Processor), a thermostatic cooler, 2 pumps (model 510), a solvent selector, and fluorescence detector (model 420-AC) all from Waters Millipore® (Toronto, Canada). A C-18 reverse phase separation column (CSC-S ODS2, 5 μm; length, 15 cm; i.d 0.46 cm; Chromatography Sciences Company, Toronto, Canada) was used as the stationary phase. The whole setup was then connected to a Digital Equipment Corp. Professional 350 computer and printer via a systems interface module. The experimental arrangement of the HPLC equipment and setup is shown in plates 1 and 2. Figure 3 shows a schematic illustration of the equipment and the connections.

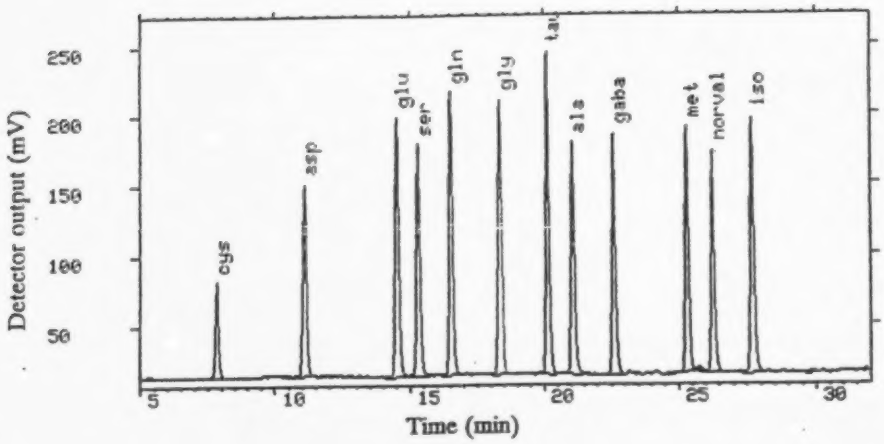
At the start of each experimental session, the buffer, methanol and double distilled, demineralized water (DDH_2O) were degassed by negative pressure filtration through 0.22 μm filters. The pumps and column were cleaned and purged of gas using water and methanol. The system was then programmed to run the mobile phase (the entire solvent gradient starting with 95% lithium acetate (LiAc) and 5% methanol; lasting about 45 min).

ii. Amino Acid Analysis.

Supernatants from the synthesis and storage experiments and perfusates from the release experiments (push-pull perfusion) were analyzed for selected amino acids using HPLC. Prior to analyses, the supernatants were appropriately diluted with 0.1 M HCl to within the range of the detector and the separation capability of the column. This was necessary because high amino acid concentrations produce less than satisfactory resolution of peaks and therefore inaccurate detection and quantification. For each sample, 180 μl of the final dilution was transfered into HPLC microsample tubes, to this was added 20 μl of internal standard (a solution containing 100 μM each of cysteic acid and norvaline; the former eluting very rapidly and serving as an indicator as to the overall performance of the HPLC setup, while the latter served as the standard against which all other amino acids were quantified, because it is not a naturally occurring amino acid, but is chemically similar to most naturally occurring amino acids and elutes distinctly from the amino acids of interest). The samples were thoroughly mixed by vortexing and set into a spring loaded HPLC vial and capped with a lid with an easily perforatable top (septum) and then set into the appropriate slot in the carrousel. 50 μl of the 200 μl solution was then sampled by the WISP for separation and quantification of amino acids. Separation of the various amino acids, based on their retention times, was achieved by using the C-18 reversed phase column as the stationary phase. The mobile phase was a solvent gradient generated with the aid of the solvent selector and a computer program, from HPLC grade methanol (MeOH) and lithium acetate (LiAc) buffered at pH 5.5. Detection of separated amino acid was achieved by monitoring column effluent with a fluorescence detector, with the sensitivity set at approximately 1.0. The excitation wavelength (λ) was 338 nm while the emission λ was set at 425 nm. Since the amino

FIGURE 4. A sample chromatogram of a standard solution containing 100 pmoles each of the amino acids of interest. A. A graph of the detected amino acids showing their order of elution and retention times. The retention times from the standard chromatogram are used as the basis for identifying identical amino acids contained in the experimental samples. Also, the resolution of the individual amino acid peaks of the standard (see B) enables quantification of the detected peaks in the experimental samples. B. A tabular summary of all the important features of each detected amino acid, such as the retention times, which is used to identify the amino acids, the area under the curve which is required for the quantification of the amino acids, and the level of response (a measure of the efficiency of detection of the amino acid) of each peak also use for quantification calculations.

A



B

```
Peak Name               Ret time      Area   Height Type    Response  Deviation    Intercept        Slope

cys                         7.83    592516    69160  BB 4.41313e+01          -   0.000e+00   4.413e-01
asp                        11.09   1342318   138514  BB 9.99775e+01          -   0.000e+00   9.998e-01
glu                        14.52   1733915   186242  BB 1.29144e+02          -   0.000e+00   1.291e+00
ser                        15.31   1554694   166849  BB 1.15796e+02          -   0.000e+00   1.158e+00
gln                        16.53   1809992   205240  BB 1.34810e+02          -   0.000e+00   1.348e+00
gly                        18.35   1781548   197080  BB 1.32692e+02          -   0.000e+00   1.327e+00
tau                        20.15   1970487   232804  BB 1.46764e+02          -   0.000e+00   1.468e+00
ala                        21.09   1521214   166374  BB 1.13302e+02          -   0.000e+00   1.133e+00
gaba                       22.61   1517593   172947  BB 1.13032e+02          -   0.000e+00   1.130e+00
met                        25.32   1458925   176311  BB 1.08663e+02          -   0.000e+00   1.087e+00
UNKNOWN                    25.74     41230     3039  BB           -          -           -           -
norval                     26.28   1342620   159008  BB 1.00000e+02          -   0.000e+00   1.000e+00
iso                        27.71   1542989   183285  BB 1.14924e+02          -   0.000e+00   1.149e+00
UNKNOWN                    29.87     36029     2714  BB           -          -           -           -
```

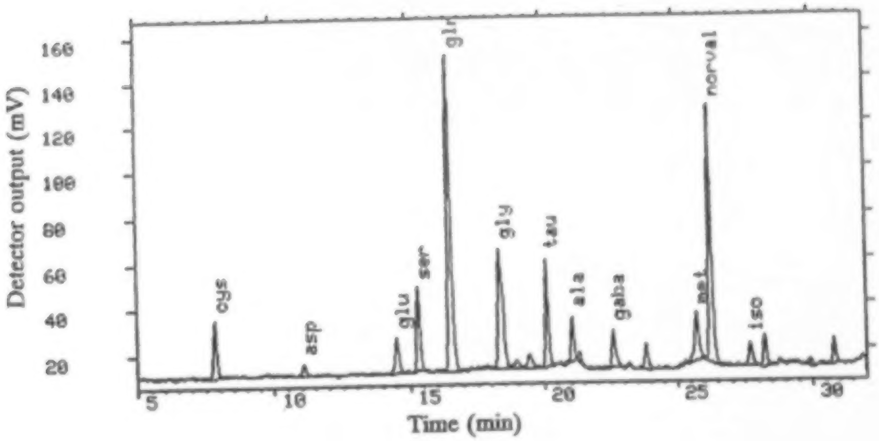
acids do not fluoresce themselves, fluorescence was achieved by precolumn derivatization with the fluorescent agent o-phthalaldialdehyde (OPA) containing β-mecaptoethanol to facilitate the derivatization process.The high pressure required to drive the mobile phase and the samples over the column (stationary phase) was produced by 2 pumps each capable of generating pressures up to 4000 pounds per square inch (psi). Pump pressures routinely rested between 2200 and 2800 psi. Quantification was then done by a computer program which converted the intensity of the fluorescence to peak size, and subsequently integrated the area under the peak. This value was then used in the following formula to calculate the total amount of amino acid in picomoles:

[area under sample peak (AUSP) ÷ area under internal standard peak (AUISP) × 100] ÷ Resolution factor (R_F).

Before any experimental samples were run, an external standard composed of 100 μM each of cysteic acid (CYS), aspartate (ASP), glutamate(GLU), serine (SER), glutamine (GLN), glycine (GLY), taurine (TAU), alanine (ALA), γ-amino butyric acid (GABA), methionine (MET), norvaline (NOR) and isoleucine (ISO)] was processed in a manner similar to the experimental samples (180 μl DDH_2O + 20 μl external standard) was run first. The 50 μl samples analyzed contained 100 pmoles of each amino acid. The retention time of this sample was then used to calibrate the computer/detector for the detection of the various amino acids in the experimental samples. Figure 4 is a sample standard chromatogram showing the order of separation, retention times, the area under the peak and the resolution factor of each amino acid. The standard chromatogram also gave a good indication as to the performance and sensitivity of the system and the suitability of the acetate buffer, since a slight change in the pH dramatically influences the separation and sharpness of the peaks. Once the characteristic chromatogram was obtained, the retention times and amount (100 pmoles) of the various amino acids were then logged into the memory forming the basis upon which any amino acid in the experimental samples could be identified. These standard retention times did not vary much from day to day as long as the buffer was fresh and properly degassed, and the pump pressure and separating columns were properly maintained and in optimal functional condition. The samples were then loaded into the carrousel sequentially in the order in which they were programmed to be run, starting first with the derivatizing agent (TAG; see composition in solution preparation) as this was always programmed as #1. A batch of up to 40 samples could be run provided adequate buffers were maintained. Each sample took about 35 min to run and was then followed by a 15 min cleanup program that preceded the next sample run. Separated amino acids were identified and quantified by the computer by integrating the area under the peak from trough to trough, and taking into consideration the R_F value of each amino acid, and also the area under the peak of the added internal standard (IS; norvaline; Fig. 5). In cases where some peaks were mislabelled or unidentified, another computer program was available that allowed retrieval of chromatograms and recalculation of the area under the peak. This value was then used to calculate the amount using the same formula that the computer uses to calculate the final amount i.e (AUSP ÷ AUISP X 100) ÷ R_F value.

FIGURE 5. A sample chromatogram of an experimental sample from rat brain perfusates, separated, identified and quantified based on information derived from the standard chromatogram (usually run daily, prior to all experimental samples). A. Computer print out of a chromatogram showing the peaks of identified amino acids and their corresponding retention times on the abscissa. B. Tabular summary of the important features of each peak (as in Fig. 4B) including the area under the peak, and the level of response that are then used to calculate the amount of amino acid present in the sample (shown in column 6). The peak height is also given, and can be used to calculate the amount of amino acid present but is much less accurate compared to calculations based on the peak area.

A



B

Peak Name	Ret time	Area	Height	Type	Amount	Intercept	Slope	Response
cys	7.82	220231	24577	BB	50.777	0.000e+00	4.413e-01	2.24087e+01
asp	11.08	48255	4734	BB	4.911	0.000e+00	9.998e-01	4.90999e+00
glu	14.51	165516	15472	BB	13.041	0.000e+00	1.291e+00	1.68414e+01
ser	15.32	334783	36956	BB	29.418	0.000e+00	1.158e+00	3.40645e+01
gln	16.52	1256668	140947	BB	94.850	0.000e+00	1.348e+00	1.27867e+02
gly	18.33	642164	52224	BB	49.242	0.000e+00	1.327e+00	6.53408e+01
UNKNOWN	18.96	43027	3243	BB	-	-	-	-
UNKNOWN	19.45	75086	5851	BB	-	-	-	-
tau	20.12	387220	47201	BB	26.846	0.000e+00	1.468e+00	3.94000e+01
ala	21.06	161959	19247	BB	14.545	0.000e+00	1.133e+00	1.64795e+01
UNKNOWN	21.30	37298	4517	BB	-	-	-	-
gaba	22.59	179938	16453	BB	16.198	0.000e+00	1.130e+00	1.83089e+01
UNKNOWN	23.82	102239	11269	BB	-	-	-	-
met	25.72	225775	20710	BB	21.141	0.000e+00	1.087e+00	2.29728e+01
norval	26.26	982792	114677	BB	Internal standard			
iso	27.70	94809	9846	BB	8.394	0.000e+00	1.149e+00	9.64690e+00
UNKNOWN	28.23	118423	14040	BB	-	-	-	-
UNKNOWN	29.90	40086	3479	BB	-	-	-	-
UNKNOWN	30.80	93742	11792	BB	-	-	-	-

iii. Catecholamine Analysis.

50 μl aliquots of prepared supernatants or appropriate dilutions from various brain regions were analysed by HPLC for catecholamines using electrochemical detection. A C_{18} reverse-phase column was used as the stationary phase while the mobile phase consisted of 0.1 M sodium biphosphate (NaH_2PO_4), 0.1 M sodium citrate, 0.1 mM ethylenediamine tetraacetic acid (EDTA), and 100 mg/l sodium octyl sulphate at pH 4.0. For each experimental run, standard samples containing 100 pmoles each of dopamine, adrenaline and noradrenaline were run first to establish the order of elution (noradrenaline < adrenaline < dopamine) and also to serve as control for quantification. The electrochemical detector was connected to a chart recorder which continuously displayed a record of the separation and peaks. The amplitude of each peak was used in the calculation of total amount of transmitter in samples by comparison with standard peak heights. The silver working electrode potential was set at 0.72V with respect to a silver-silver chloride (Ag-AgCl) reference electrode. At this potential, the contribution of any sulfide ions that may be present was negligible as Goodwin *et al.* (1988) had shown sulfide oxidation potential to be maximal at the much lower potentials of 0.00 - 0.06V.

II. ELECTROPHYSIOLOGICAL STUDIES

a. Sucrose-Gap Recordings in Frog Sympathetic Ganglia

i. Dissection and tissue preparation.

"Northern" leopard frogs (*Rana pipiens*) were pithed and the abdomen opened and cleared of internal organs. The VIth to Xth paravertebral ganglia together with the rostral portion of the VIIIth spinal nerve, the sympathetic chain and a short portion of the IXth or Xth spinal nerve were then removed and excess connective tissue carefully dissected out under a dissection microscope.

The preparation was then mounted in a sucrose gap recording chamber (Smith, 1984). The preganglionic fibre was placed in the first compartment and submerged in mineral oil. The IXth or Xth paravertebral ganglia were placed in the main compartment of the recording chamber and continuously superfused with Ringer's solution [composition in mM: NaCl, 100; KCl, 2; $CaCl_2$, 1.8; tris (hydroxymethyl) aminomethane-HCl pH 7.2, 16; and D-glucose 10]. One *ramus comminicans* containing the postsynaptic axons was placed across a second compartment of the chamber and superfused with isotonic sucrose. A short stub of the IXth or Xth spinal nerve was placed in a third compartment containing isotonic KCl solution. The various solutions in these compartments were separated by petroleum jelly seals. The compartment containing the ganglion was grounded by means of a Ringer-agar bridge connected to a calomel electrode. A similar calomel electrode-Ringer-agar bridge arrangement connected the chamber containing the postganglionic fibres to an electrophysiological recording system. Responses were recorded on a rectilinear pen recorder (Gould-Brush model 2400), frequency response filtered at 5 Hz. Ganglionic depolarizations were indicated by upward pen deflexions while hyperpolarizations were indicated by downward pen deflexion.

ii. Recording

Changes in membrane potential produced by various agonists and hydrogen sulfide were record from frog IXth and Xth paravertebral sympathetic ganglia by the sucrose gap technique. Frog Ringer's and drugs (dissolved in Ringer) drained from overhead reservoirs under gravity to superfuse the tissue. The pH of NaHS in frog Ringer's was 7.5. This pH was not expected to influence the effect of sulfide as Rafuse *et al.* (1988) had shown that frog Ringer's solution at pH 7.55 did not alter any of the responses in sympathetic ganglia. The ganglia were constantly superfused with ringer and a three-way switch valve allowed addition of drugs contained in other reservoirs. The agonists used to induce changes in membrane potentials were: adrenaline, 1 μM; muscarine, 10 μM; and nicotine, 10 μM. Each drug was applied for 30 sec and 15 min was left between successive drug applications to avoid desensitization. For the Na^+/K^+ pump experiments, a Ringer's solution low in K^+ (0.2 mM) was used to inactivate the pump and normal-K^+ (2.0 mM) Ringer's solution was applied to reactivate it (Smith *et al.*, 1986). The normal-K was applied for 90 sec at a regular interval of 15 min. This allowed for reproducible, pump-mediated potassium activated hyperpolarization responses. 1.8 mM NaHS (Aldrich) in Ringer's solution was used in all experiments. This concentration is one fifth that used in other *in vitro* electrophysiological experiments (Warenycia *et al.*, 1989). The experimental paradigm used in all cases was to obtain 2 control responses, one response in NaHS and 2 or 3 recovery responses. In another set of experiments, NaCl was replaced by equimolar sucrose.

iii. Data Analysis:

All responses were normalized to the control response immediately preceding sulfide treatment and reported as percent of control response ± SEM. The control response, the sulfide response and the first recovery response were compared using the Duncan's Multiple-Range Test (Dowdy and Weardon, 1983) and $p < 0.05$ was considered significant. Only data from experiments demonstrating substantial recovery from sulfide treatment were included in the analysis.

b. Intracellular Recording in Dorsal Raphe (DR) Nucleus

i. Dissection and Preparation of Brain Stem Slices.

Male Sprague-Dawley rats (200-350g) were put under light halothane anaesthesia and killed by a heavy blow to the spinal cord. The brain was then quickly dissected out and placed on ice-cold carbogenated (95% O_2 and 5% CO_2, pH=7.35-7.4) artificial cerebrospinal fluid (ACSF). The brain was then transferred unto a saline-soaked filter paper laying on an ice-parked petri dish and carefully cleaned of the dura. The overlying cerebral cortices and hippocampi were gently parted at the midline and the *corpus collosum* cut. These cortical structures were then peeled back to expose the colliculi. A single edge razor blade was then used to block off the midbrain region containing the medulla and pons. Prior to dissection, a 3% agar gel was cut and glued to the back of a tissue holder to serve as both a support for the tissue and to protect the blade from the hard perspex backing. The agar was then trimmed vertically and a puddle of liquid cyanoacrylate adhesive (superglue) applied

PLATE 3

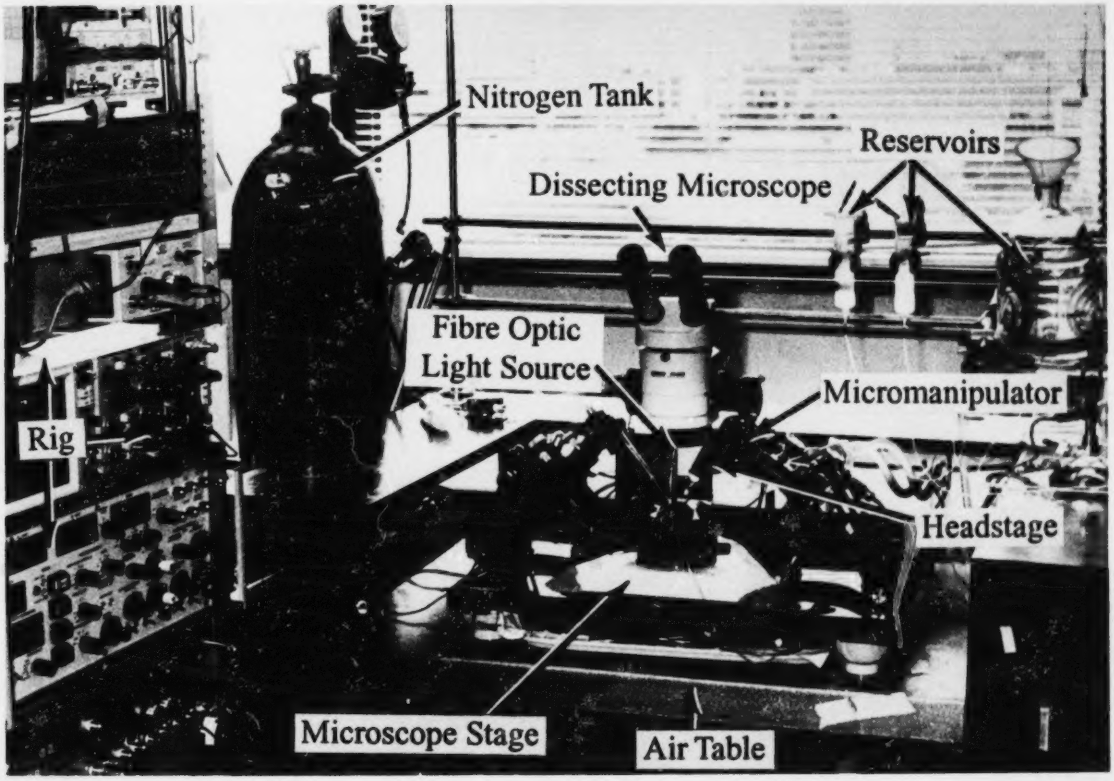

Nitrogen Tank
Reservoirs
Dissecting Microscope
Fibre Optic Light Source
Micromanipulator
Rig
Headstage
Microscope Stage
Air Table

Plate 3 Experimental apparatus used for electrophysiological recording of the dorsal raphe nucleus.

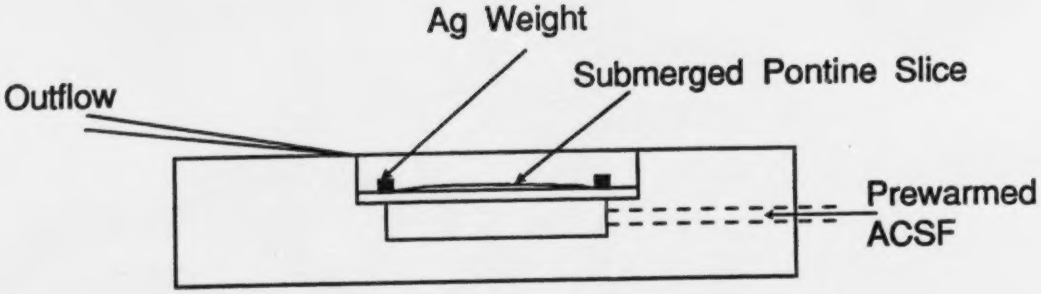

Ag Weight
Submerged Pontine Slice
Outflow
Prewarmed
ACSF

FIGURE 6 The "Scottish submersion chamber" used for *in vitro* electrophysiological recording of the dorsal raphe nucleus of the rat brain stem slice (Adapted from Alger et al., 1984)

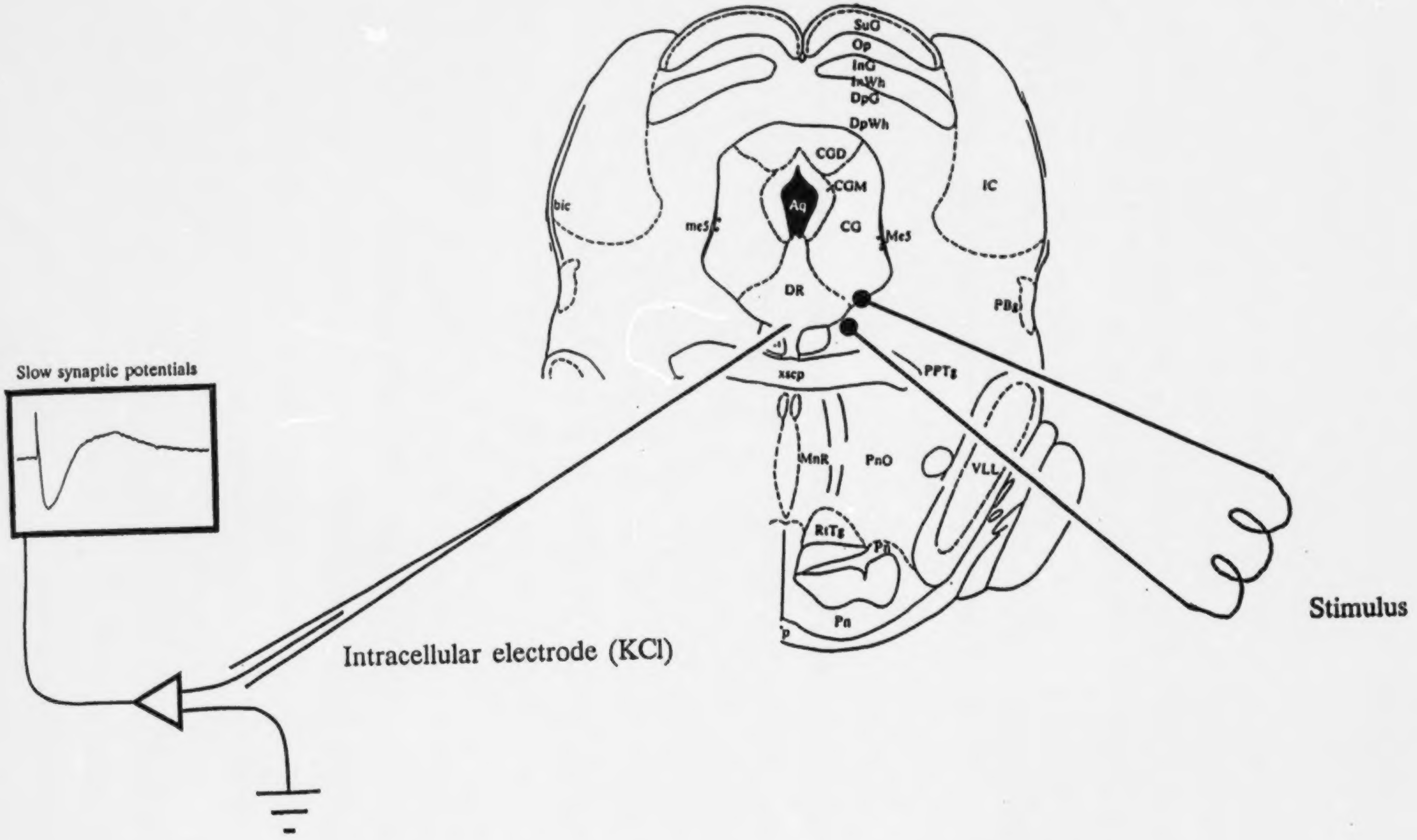
to the base of the tissue holder. A wedge of dry filter paper was used to pick up the blocked brainstem and place it rostral end down on the tissue holder onto the glue puddle, such that the ventral surface rested securely against the agar. The holder, with the tissue in place was then quickly clamped into the vibratome and the chamber was then filled with ice-cold (4°C) carbogenated ACSF. Coronal sections were produced from caudal to rostral, with cutting proceeding from the dorsal surface to the ventral surface. 350 μm thick coronal sections were then cut from the pontine region containing the DR. Slices were taken from regions corresponding to the opening of the aqueduct down to the disappearance of the cerebellar peduncles. The slices were immediately placed in a solution of carbogenated ACSF and incubated at 34°C for 1 hour. A slice was then transfered to a recording chamber where it was completely submerged and continuously superfused, at a rate of 2.5-3 ml/min, with carbogenated ACSF, which had been prewarmed to 34 ± 0.2°C, (see below). The composition of the ACSF was (in mM): NaCl, 126; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; $CaCl_2$, 2.4; D-glucose, 11; $NaHCO_3$, 25.

ii. Slice Chamber and Recording Station.

Plate 3 shows the recording station used in these studies. The entire setup for intracellular recording was mounted on a steel plate placed on tennis balls on top of an air isolation table (Micro-G). The recording chamber was mounted on a stage of a Wild M3Z dissecting microscope, and illuminated from below, via an adjustable mirror, with a fiber optic light source. The recording chamber, machined from a 9

FIGURE 7. A schematic diagram of the rat pontine slice, containing the dorsal raphe nucleus, showing the placement of the stimulating and recording electrodes. The aqueduct (Aq) is an important anatomical marker for locating the dorsal raphe nucleus in coronal section.

Rat Pontine Slice


SuG
Op
InG
InWh
DpG
DpWh
CGD
CGM
IC
bic
me5
Aq
CG
Me5
DR
PBg
xscp
PPTg
MnR
PnO
VLL
RtTg
Pn
Slow synaptic potentials
Intracellular electrode (KCl)
Stimulus

cm by 4 cm piece of 3/16" thick plexiglass, is similar to the one developed by Henderson and Williams (the "Scottish submersion chamber"; Alger *et al.*, 1984; Williams *et al.*, 1988; Fig. 6). Briefly, the chamber consists of a circular well drilled into the plexiglass. Within this is suspended, with the aid of tiny flexible wedges, a tissue grid made by gluing nylon meshing onto a 1.5 mm thick ring cut from a 5 ml plastic syringe. The volume of the chamber is about 500 μl. Slices were placed on the mesh with a strip of lens paper backing. An electron microscope (EM) grid was then placed over the region corresponding to the DR (see Fig. 7). Both the slice and grid were then held in place with tiny pieces of flattened silver wire. ACSF was prewarmed in a one liter reservoir, and later in a 60 ml syringe jacket, by a thermostatically controlled (40°C) water circulator. The reservoir was also constantly bubbled with carbogen to oxygenate the medium and to maintain the pH. A polyethylene (PE-160) tubing led from the reservoir to a 4-way HPLC (Hamilton) valve, which permitted a rapid, low dead volume switching between source reservoirs. Two other reservoirs (made from the barrels of 60 ml plastic syringes) were also connected to the valve via PE-160 tubing. ACSF drained by gravity via a PE-100 tubing through a water jacket which was warmed to 40°C to the chamber. The flow rate was maintained at a constant rate of 2.5-3 ml/min. The flow rates of all reservoirs were carefully adjusted to the same rate (by altering the overhead pressure) at the beginning of each day to avoid sudden flow rate changes following switching. A change of ACSF at the HPLC valve took about 20-40 sec to fill the bath. ACSF entered the bath from the rear, below the mesh, and flowed around, beneath and over the slice, which was then submerged at a depth of about 1 mm in bathing solution. A 2-3 mm wide and 2-3 cm long wick of paper (cut from Kimwipes®) was placed at the front of the chamber to ensure a constant fluid level; one end of the wick was draped over an Ag-AgCl pellet which served as the chamber ground. A 21 gauge needle, attached to a siphon tube made of Tygon® R3603 tubing, was placed on the wick , near the front edge of the chamber. ACSF drained by gravity from the wick into a collecting bucket below by siphon action.

A bipolar stimulating electrode, made from two electrolytically sharpened, glass-insulated tungsten rods and cemented into the barrel of a pasteur pipette, was held in a Narishige® MP-2 mechanical micromanipulator which permitted selective placement of the electrode into the required region in the slice. An amplifier headstage was mounted onto another Narishige® MP-2 (Tokyo, Japan) mechanical micromanipulator which allowed 3-dimensional movement. Both manipulators had magnetic bases which permitted them to be firmly fixed to the steel plate. Microelectrode holders were plugged directly in to the headstage. The headstage and the entire setup was then carefully grounded to minimize electrical interference (See plate 3).

iii. Conventional Microelectrode Recording

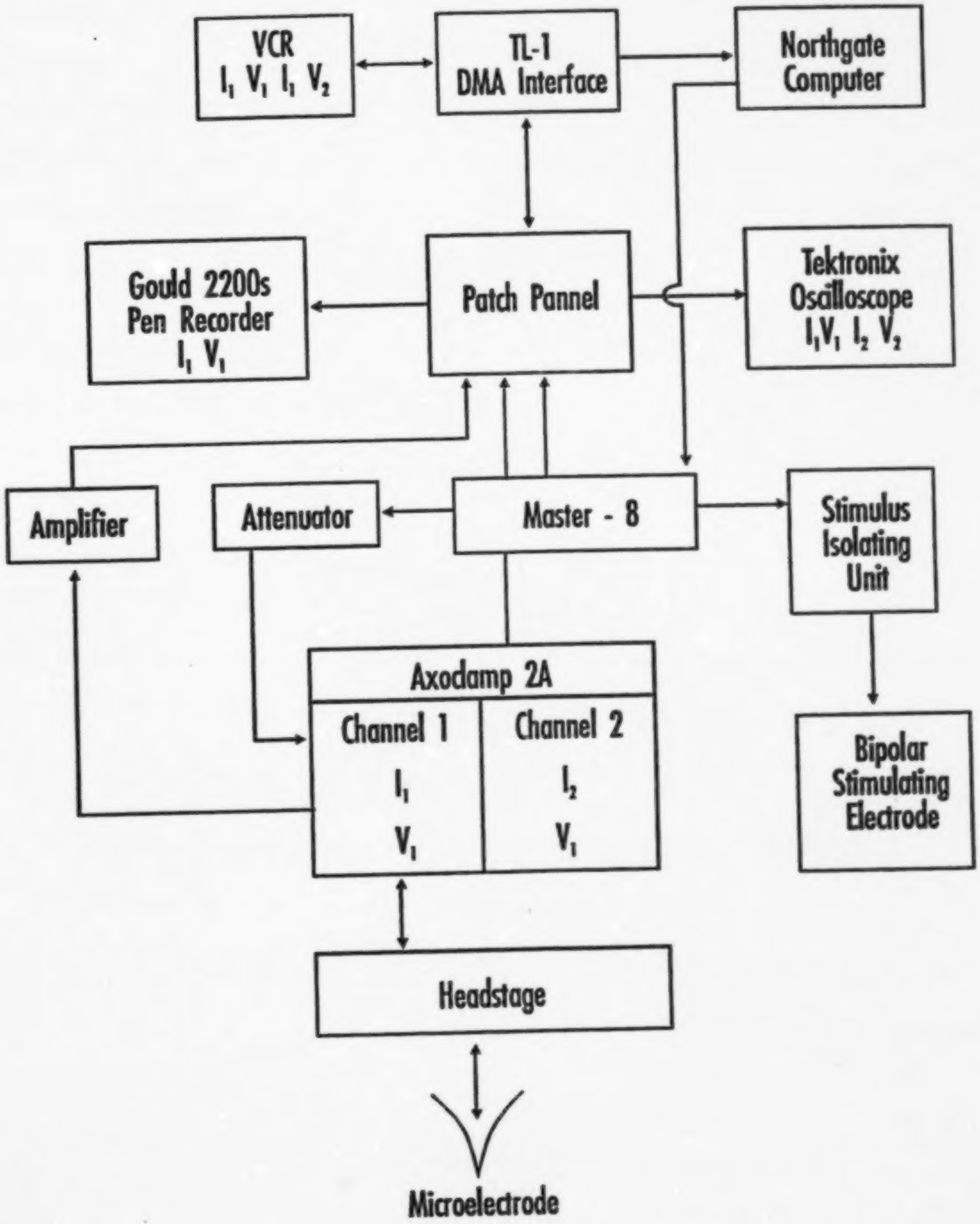
The experimental arrangement of electrodes for recording is shown in plate 3. The arrangement of the electrodes in the slice is shown in figure 7. Since the neural inputs and networks of the DR nucleus have not been clearly elaborated and/or are not structured orderly as in the hippocampus (Dingledine, 1984), the stimulating electrode was placed in or near the nucleus. This allowed focal stimulation of unspecified pathways, but which nevertheless triggered synaptic responses which could be recorded in impaled cells (Williams *et al.*, 1988). Stimuli were generated through a stimulus isolating unit (A.M.P.I., Jerusalem, Israel). Stimulus intensities of 10-40V were routinely used, with the voltage clamp experiments tolerating only stimulus intensities of no more than 25V. Stimulating voltages were chosen such as not to elicit APs and only submaximal voltage strengths were used during experiments.

Intracellular microelectrodes were pulled by a Flaming/Brown Micropipette Puller (model P-87; Sutter Instruments, U.S.A) from 1 mm diameter, regular wall filamented glass (World Precision Instruments, Inc. New Haven, CT) and filled with filtered 2M KCl. These electrodes had direct current (d.c.) tip resistance of 50-120 MΩ. Cells used for experiments satisfied the following criteria: had resting membrane potentials (RMP) of -60 to -75 mV; were stable for at least 15 min after impalement; threshold for firing action potentials (AP) of 40 to 50 mV and an AP "overshoot" of 25 to 35 mV, and duration of approximately 4-5 msec with no rapid depolarising afterpotentials. Cells with the latter two characteristics often fired spontaneously, with very narrow (< 1 msec duration) APs which are characteristic of interneurons (Schwartzkroin and Mathers, 1979). Finally, cells must also have demonstrated the characteristic complex synaptic response (Pan and Williams, 1989) and show sensitivity to the 5-HT$_1$ receptor agonist 5-carboxamidotryptamine (5-CT) with a hyperpolarization to be considered serotonergic.

The microelectrodes were back filled with 2M KCl, ensuring complete tip filling and placed into the holder, which was then slipped into the headstage that was clamped firmly onto the manipulator. The headstage was connected to channel 1 of an Axoclamp 2A amplifier, and used in the bridge current clamp mode. Bridge balance was closely monitored throughout current clamp experiments. Cell acquisition was through a program on a Master-8 (A.M.P.I.; Jerusalem, Israel), an 8 channel programmable pulse generator. Once a cell was acquired, another program was used to generate direct current pulses to the microelectrode, via the amplifiers to the bridge circuit, or to trigger voltage pulses to the bipolar electrode, via the stimulus isolation unit. The Master-8 could be triggered either internally or through an external triggering output generated by a computer (Northgate Elegance 386/20, Plymouth, MN) running a pClamp software (Axon Instruments, Burlingame, CA). Single or paired (interstimulus interval, ISI =20 msec) pulses of appropriate intensity and duration of 1-2 ms were routinely employed. Following application of drug, synaptic potentials were evoked only after the membrane potential (V_m) had returned to the original level with DC current via the balanced bridge, thus ensuring equal driving force for membrane currents in control and in the presence of drug.

Voltage signals were delivered to a patch panel from which they were connected via an Axon Instruments• analog to digital convertor (ADC, TL-1 DMA Interface; Foster City, CA; Fig. 8) and to a Vetter model 200-T PCM (Rebersberg, PA) data recorder for off-line analysis with a Nicolet 4094 digital oscilloscope

FIGURE 8. A schematic diagram of the experimental arrangement of the equipment used for intracellular recording in the dorsal raphe nucleus. (VCR = video cassette recorder, Toshiba Digital 4; Master-8 = pulse generator; Axoclamp 2A = amplifier, TL1 DMA Interface = analog to digital and digital to analog converter).


VCR
I_1 V_1 I_1 V_2
TL-1
DMA Interface
Northgate
Computer
Gould 2200s
Pen Recorder
I_1 V_1
Patch Pannel
Tektronix
Oscilloscope
I_1V_1 I_2 V_2
Amplifier
Attenuator
Master - 8
Stimulus
Isolating
Unit
Axoclamp 2A
Channel 1
I_1
V_1
Channel 2
I_2
V_1
Bipolar
Stimulating
Electrode
Headstage
Microelectrode

(Mississauga, Ont.) and to a computer for on-line analysis. A DC-coupled pen chart recorder (Gould recorder 2200S; Cleveland, Ohio) also drew a signal from the panel and displayed a continuous record of all the voltage and current signals. All evoked potential data on computer were digital averages of 3 successive responses taken a minute apart. In each response, the clock was slowed down 8 fold, after the sEPSP had peaked to capture the return to resting membrane potential.

For voltage clamp experiments, the discontinuous single electrode voltage clamp (SEVC) technique was employed. For these experiments, the electrodes were shielded with sylgard® (Dow Corning; Midland, Mich.) to reduce capacitance. Following cell acquisition in the bridge circuit mode, the amplifier was switched to the discontinuous current clamp (DCC) mode and the RMP balanced. In this mode, it was also possible to examine the electrode tip characteristics (by passing current, usually hyperpolarizing current) on another oscilloscope (Hitachi V212, 20 MHz; Hitachi Denshi Ltd, Japan) and hence to adjust the clamp characteristics to obtain a stable voltage clamp. To achieve this, the switching (sampling) frequency was set at 3.8-4.0 KHz and the time constant at 0.2 msec. The capacitance compensation, the anti-alias filter, the phase shift, and the output gain (0.7-1.5 nA/mV) were then carefully adjusted to obtain the most stable, optimal clamp. This corresponded to a nearly square headstage voltage waveform corresponding to a complete decay of each voltage sample before the next sample was taken. The amplifier was then switched into the SEVC mode with a holding potential at or near rest. During experiments, neurons were always held at -60 mV (V_h). At this voltage, the above parameters were again adjusted to yield the most stable clamp. The output signal was filtered at 0.1-3 KHz depending on the noise level of each clamp and was kept constant throughout an experiment.

Three types of experiments were performed in voltage clamp mode: *a*. synaptic responses were acquired in a manner similar to the synaptic potentials in current clamp. *b*. ramp experiments where the voltage was slowly (over a period of 2 sec) ramped from V_h (-60 mV) to -125 mV, and held at this potential for a further 2 sec to stabilize the membrane, then ramped slowly (over a period of 20 sec) to -40 mV. This was repeated 30 sec later and the two responses digitally averaged and stored. *c*. step-activated experiments in which 8 voltage steps of equal increments (-10 Mv) and 1s in duration were triggered from V_h to potentials negative and positive to V_h. The corresponding instantaneous and steady-state currents at every test voltage (V_t) were digitized and stored for analysis (I-V plots).

iv. Whole Cell Recording (WCR).

The discovery of the patch clamp technique by Neher and Sakmann in 1976 permitted the first recordings of currents from single ion channels. Using novel circuitry and a low resistance, fire-polished micropippettes, they demonstrated that an electrical seal could be formed (in the order of 50 MΩ), by a strong chemical interaction, between the muscle membrane and a heat polished glass pipette. The magnitude of this seal significantly improved the signal-to-noise ratio. Also, the accuracy of current measurement could be enhanced by improving the seal between the glass pipette and the membrane. Sigworth and Neher (1980) described a technique for achieving this improved seal. They observed that the application of a small amount of negative pressure to the interior of a polished glass pipette (2-5 MΩ tip resistance) resulted in the formation of electric seals, in the order of 10-100 GΩ, upon contact with many biological membranes. Because the resistance of this seal was in the gigaohm (10^9 Ω) range, it was referred to as the "giga-seal technique". It was subsequently found that rupture of a cell-attached patch after the formation of the gigaseal permitted high quality intracellular recordings in current and voltage clamp (Hamill *et al.*, 1981). The high resistance seal prevented current from flowing between the membrane and the electrode, and the edge of the pipette aperture and ensured low electrode resistance, providing optimal conditions for voltage clamp of the cell. This therefore permitted voltage-clamp studies of cells that were hitherto, not amenable to intracellular recording due to their size and inability to withstand the trauma of impalement. The formation of the "giga-seal" on the membrane also afforded a high degree of mechanical stability which enabled other recording configurations, with their accompanying advantages, to be achieved by simple manipulations; namely cell-attached patch, whole-cell recording (WCR), outside-out patch and inside-out patch (Hamill *et al.*, 1981). The WCR mode of recording particularly permitted the examination of macrocurrents in cells and the role of various macromolecules in signal transduction and their modulation of channel activity. The easy access to the interior of the cell allowed these substances or their antagonists to be easily applied intracellularly. However, it has the disadvantage that diffusible endogenous macromolecules exchange easily with pipette contents leading to their dilution or removal from the cell. This can contribute to the phenomenon called "run down" where electrophysiological properties of the cell disappear with time (Fenwick *et al.*, 1982; Doroshenko *et al.*, 1982; Neher, 1988). Therefore, recordings in this mode generally did not last long in comparison to microelectrode impalements.

Some of the experiments in this thesis were done using a slight modification of the whole-cell recording mode, the "slice-patch" (Blanton *et al.*, 1989; Coleman and Miller, 1989)

v. Patch Electrode Preparation.

The WCR electrodes were made from thin wall borosilicate glass tubing, 1.5 mm outer diameter (OD) and 1.17 mm inner diameter (ID) (World Precision Instruments Inc.; Sarasota, FL). The electrodes were pulled on a double-stage pipette puller (Narishige PP-83; Tokyo, Japan) in two stages, yielding gradually tapered micropipettes with a tip aperture approximately 1-2 μm and resistance between 5-8 MΩ.

vi. "Slice-patch" Whole-Cell Recording.

When the "giga-seal" whole-cell recording was described (Hamill *et al.*, 1981), it was thought to be applicable only to enzymatically dissociated or cultured cells that had little extracellular matrix to clog the tip and that could be visualized and hence directly approached. It was thought impracticable to apply this technique in slice preparation to study neurons or excitable tissue *in situ* or to examine synaptic events mainly because the normal extracellular matrix which would prevent gigaseal formation that is crucial to this technique. However, this has recently been achieved in amphibian retina neurons (Coleman and Miller, 1989) and in reptilian and mammalian cortical neurons (Blanton *et al.*, 1989). Some of the experiments described in this thesis were done using a technique identical to those in the above two studies, in the brain stem slice preparation.

Low resistance glass electrode pipettes (5-8 MΩ), were back-filled with an intracellular recording solution with following composition (in mM) K-gluconate, 145; $MgCl_2$, 2; HEPES, 5; EGTA, 1.1; $CaCl_2$, 0.1; Na-ATP, 5; Na-GTP, 0.3; adjusted to pH 7.2 with KOH, osmolarity between 270 -285 mosm. This solution was filtered through a 0.22 μm filter into 5 ml sample tubes and frozen at -80°C and used within 3 days.

The filled electrode was inserted into an electrode holder (EH-2MSW, 1.5; E.W Wright; Guilford, CT) that provided an electrical connection to the clamp amplifier via the headstage and a right angle port for a suction line. A silver wire in the holder was chlorided daily by dipping it into molten silver chloride producing a silver-silver chloride wire. The glass electrode was inserted, the holder tightened and inserted into the headstage as for sharp electrode recordings. A small positive pressure was applied via the port (3-4 ml volume displacement of a 10 cc syringe connected to the open end of the vacuum line). The electrode was then advanced with short, sharp movements towards the submerged slice preparation and the electrode resistance monitored by applying a 20 msec, -0.12 nA intermittent current pulse (interpulse interval of 50 msec; ≈20 Hz) to its tip. Whenever the electrode came into contact with a cell membrane, the apparent electrode resistance increased. The positive pressure was then relieved and an additional gentle suction (either by displacement of 3-5 ml volume of the syringe or by mouth) was applied if necessary, which often increased the resistance of the cell-electrode seal to the gigaohm range (1-10 GΩ). This seal often formed instantly or took 15-120 sec to completely form and stabilize. Once the seal was stabilized, additional gentle suction, by mouth, was applied to rupture the membrane patch; this resulted in a marked drop in the resistance and revealed the resting potential of the cell. More often than not, a small puff of positive pressure was applied at this stage to improve the access to the intracellular milieu. Following this puff, the resistance again dropped slightly.

Once acquired, the condition and suitability of the cell was assessed by determining its RMP, action potential threshold and overshoot. In contrast to conventional sharp microelectrode impalement, cells acquired by this technique rarely required time or a hyperpolarizing current to recover from the trauma of impalement and to improve their responsiveness, but instead, were at their best soon after acquisition and responses actually deteriorated with time, possibly from the "run down" phenomenon. The formation of a seal with each electrode was attempted at the most twice. Electrodes were discarded if a seal was not obtained on the second pass.

For voltage clamp experiments, the continuous single electrode voltage clamp (SEVC) mode of the Axoclamp was used. The anti-alias filter was turned off throughout the experiment. The amplifier gain routinely rested between 3-8 nA/mV while a time constant of 20 msec was used (throughout this study) to yield a stable clamp. Fast and slow pipette capacitance, whole cell capacitance and series resistance were completely compensated for using the gain and phase lag circuits and bridge circuit to optimize the response to 10 mV, square-wave voltage steps. As with sharp electrode records, all currents were filtered at 3-10 kHz, digitized and stored on tape or computer. Also a continuous pen chart record was kept of all experiments (Gould Recorder 2200S; Cleveland, Ohio).

vii. Data Analysis

Changes in resting membrane potentials or current were measured from resting level in ACSF to the maximum amplitude seen in the presence of drug or HS^- application. The washout hyperpolarization or its corresponding outward current was calculated as the maximum change from the level at which washing began to the peak of the response. Synaptic currents or potentials were measured as the maximum amplitude from rest (V_m) to the peak of the response. For the ramp experiments, the first 4 sec (ramp down to -115 or -125 mV and stabilization period) and the return to V_b were deleted in all analysis. The voltage was appropriately adjusted for electrode offset before subtractions were done (mainly insets). In step experiments, only peak steady-state currents were measured and used to plot current-voltage (I-V) curves.

Statistics: Only data obtained from neurons which showed substantial recovery (> 90%) from HS^- and other drug effects were included in analysis. All data are presented as mean ± standard error of the mean (SEM). Neurons were used as their own control for statistical comparison using the paired Student t-test. The control response immediately preceding drug application was compared with the response in the presence of drug or HS^-. Statistical tables (Wonnacott and Wonnacott, 1972) were used to determine probability values; $p<0.05$ was considered significant.

viii. Construction of Dose-Response Curves

At pH 7.45, whether gaseous H_2S or the NaHS was originally the source, approximately one-third exists as H_2S and two-thirds as HS^- (Beauchamp *et al.*, 1984). The concentration of HS^- applied to the slices was based on those found in the brains of rats following acute exposure to H_2S gas or IP injection of a solution of NaHS. At the LD_{50}, brain acid-labile S^{-2} was determined to be about 75 μM (Warenycia *et al.*, 1989; Goodwin *et al.*, 1989). For the construction of dose-response curves therefore, concentrations of between 30-1000 μM were applied for 3-6 min to ensure an equilibrium response. To further elucidate the mechanism of action of HS^- two doses, either 150 or 200 μM was applied. 200 μM HS^- reduced the IPSP and DSC by approximately 75-80% while completely abolishing the sEPSP. Following HS^- application, the preparation was washed in ACSF for about 10-15 min during which substantial recovery (>95%) occurred. Fresh stocks of NaHS solution were made once or twice daily, from which appropriate dilutions were made in carbogenated ACSF to a final volume of 50 ml immediately prior to bath application. Each dilution was used only once and the remainder discarded as this solution is known to lose potency on extended exposure to air. Although we attempted to construct complete dose-response curves for each neuron, this was not usually possible.

III. *IN VIVO* TOXICITY STUDIES.

The toxicity profile of sulfide was determined in rats using NaHS. IP injections of various doses of a solution of NaHS were administered to groups of male Sprague-Dawley rats. Rats were randomly divided into groups, each receiving a dose of sulfide. Following sulfide injection, the animals were continuously monitored for 30 min initially, and then observed 24 hours later to determine survival. Rats that were alive after 24 hours were considered survivors. Nine sulfide concentrations of between 5-40 mg/kg were selected for this study. The LD_{50} was then determined by the cumulative method of Reed and Meunch (Turner, 1965). This method allowed fewer number of animals to be used per concentration of drug while still attaining high ***n*** values (sample size). Briefly, it is presumed that any animal surviving a higher doses of a drug will also survive the lower doses and so is counted among the survivors of all doses below it. Conversely, non-survivors (dead) of lower doses are presumed will not survive the higher doses and are therefore counted among the dead of the doses above it. Once this was tabulated, a graph of percent mortality versus log dose was plotted, from which the LD_{50} was determined. The same method was used in the determination of the LD_{50} of dithiothreitol (DTT).

The ability of DTT to protect rats or reverse the intoxication following poisoning was examined *in vivo* by administering the LD_1 of DTT at specific times prior to, or post exposure to sulfide. The LD_1 of DTT was determined by linear extrapolation from the concentration-lethality curve.

IV. PREPARATION OF SOLUTIONS.

a. Artificial Cerebrospinal Fluid (ACSF):

10 X stock solutions were prepared by dissolving all the salts except $NaHCO_3$ and D-glucose in "Milli Q" (Millipore, Mississauga, Ont.) or DDH_2O. 1 in 10 dilutions of these stocks were made daily and the appropriate amount of $NaHCO_3$ and D-glucose then added. For *in vitro* experiments, the final solution was bubbled with carbogen that buffered the solution at pH 7.3 - 7.4.

b. HPLC: External Amino Acid Standards.

A stock of amino acid standards containing 100 μM each of the following amino acid: glutamic acid, glutamine, glycine, taurine, γ-amino butyric acid, methionine, norvaline, isoleucine, aspartic acid, cysteic acid, serine, and alanine was prepared by dissolving in DDH_2O. This solution was then stabilized by incorporating 500 μM ascorbic acid. 2 ml aliqouts were pipetted into sample tubes and frozen at -30°C until use. The solution was thawed when required and a 1 in 5 dilution made to produce a 20 μM solution. A 1 in 10 dilution of the latter solution (2 μM) was then applied to the HPLC for analysis, so that the 50 μl sampled for analysis contained 100 pmoles of each amino acid.

The internal standard was prepared in a similar manner except that this solution contained only norvaline and cysteic acid. It was also stabilised with 500 μM ascorbic acid.

c. Derivatizing Agent (TAG):

This solution contained 20 mg of o-phthalyldialdehyde (OPA), 50 μl of β-mercaptoethanol, 75 μl BRIJ 35*, all in 25 ml of borate buffer. Because OPA is not very soluble in the buffer, it was first dissolved in 500 μl of HPLC grade methanol before incorporation into the buffer. TAG was prepared fresh daily and stored in the dark until use.

d. Borate Buffer:

0.4 M borate buffer was made by dissolving 12.37 g of boric acid in 450 ml of DDH$_2$O. The suspension formed in water was stirred vigorously for about an hour to get the acid into solution. The pH was then adjusted to 10.40 with 45% KOH and made up to 500 ml with DDH$_2$O.

e. Lithium Acetate (LiAc) Buffer:

A 0.1 M solution of LiAc buffer was prepared by dissolving 20.40 g of LiAc in 1.5 l of DDH$_2$O. The pH was then adjusted to 5.50 with 1 M HCl and made up to 2 l. This remained good for a week if stored refrigerated.

f. Intracellular Recording Solution:

25 ml of intracellular recording solution was prepared by weighing the appropriate amounts of salts or aliquoting the appropriate volume of stocks into a small beaker. To this was added about 15 mls of "Milli-Q" and dissolved. The pH of the solution was then determined (routinely between 5-5.5) and titrated to pH 7.2 with 1 M KOH. The solution was then transfered into a 25 ml volumetric flask and made up to volume with "Milli-Q". Minute portions of the final solution were then pipetted and the osmolarity determined in triplicate using a micro-osmometer model 3MO (Advanced Instruments Inc.; Needham Heights, MA) and adjusted, if necessary with "Milli-Q" to between 270-285 mosm. The final solution was then filtered through a 0.22 μm filter into 5 ml sample tubes and frozen at -80°C.

g. Others:

All drugs were made by dissolving in "Milli Q" or DDH_2O to produce hypertonic solutions. Appropriate aliquots were then sampled and added to the physiological solution to produce the final concentration. Strophanthidin, which is not soluble in water, was dissolved in 100% undenatured ethanol. All stock drug solutions were stored in a refrigerator until required.

V. DRUGS AND THEIR SOURCES.

γ-amino butyric acid (GABA; Sigma, MO)

2-mercaptoethanol (BDH, England)

2-methyl butane (Fisher, NJ)

Adenosine 5'-triphosphate (disodium salt, ATP; Serva, N.Y.)

Barium chloride (Sigma, MO)

Boric acid (Fisher, NJ)

Cadmium chloride (Sigma, MO)

Calcium chloride (Fisher, NJ)

Cesium chloride (Aldrich, WI)

D-glucose (BDH, Toronto)

D-methionine (Sigma, MO)

D-norvaline (Sigma, MO)

DL- glutamic acid (Sigma, MO)

DL-aspartic acid (Sigma, MO)

Dopamine (Sigma, MO)

Ethyl carbamate (urethane, Pfaltz and Bauer)

Ethyleneglycol-bis-(β-amino ethyl ether) N,N'-tetra acetic acid (EGTA; Sigma, MO)

Formalin phosphate 10% buffered (Fisher, NJ)

Glycine (Sigma, MO)

Guanosine 5'-triphosphate (sodium salt; GTP; Sigma, MO)

Halothane (Aldrich, WI)

Hydrochloric acid (Fisher, NJ)

L-alanine (Sigma, MO)

L-ascorbic acid (Fisher, NJ)

L-cysteic acid (Sigma, MO)

L-glutamine (Sigma, MO)

L-isoleucine (Sigma, MO)

L-noradrenaline bitartarate (Winthrop)

L-serine (Sigma, MO)

Lithium acetate (Sigma, MO)

Methanol, HPLC grade (Fisher, NJ)

N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES; BDH, Toronto)

± Nipecotic acid (Sigma, NJ)

O-phthaldialdehyde (OPA; Sigma, MO)

Polyoxyethylene 23-lauryl ether (BRIJ® 35; Sigma, MO)

Potassium chloride (Fisher, NJ)

Potassium hydroxide (Fisher, NJ)

Sodium hydrogen phosphate (BDH, Toronto)

Sodium phosphate (Fisher, NJ)

Sodium chloride (Fisher, NJ)

Sodium hydrosulfide (Aldrich, WI)

Taurine (Sigma, MO)

Urea (Fisher, NJ)

CHAPTER III

RESULTS

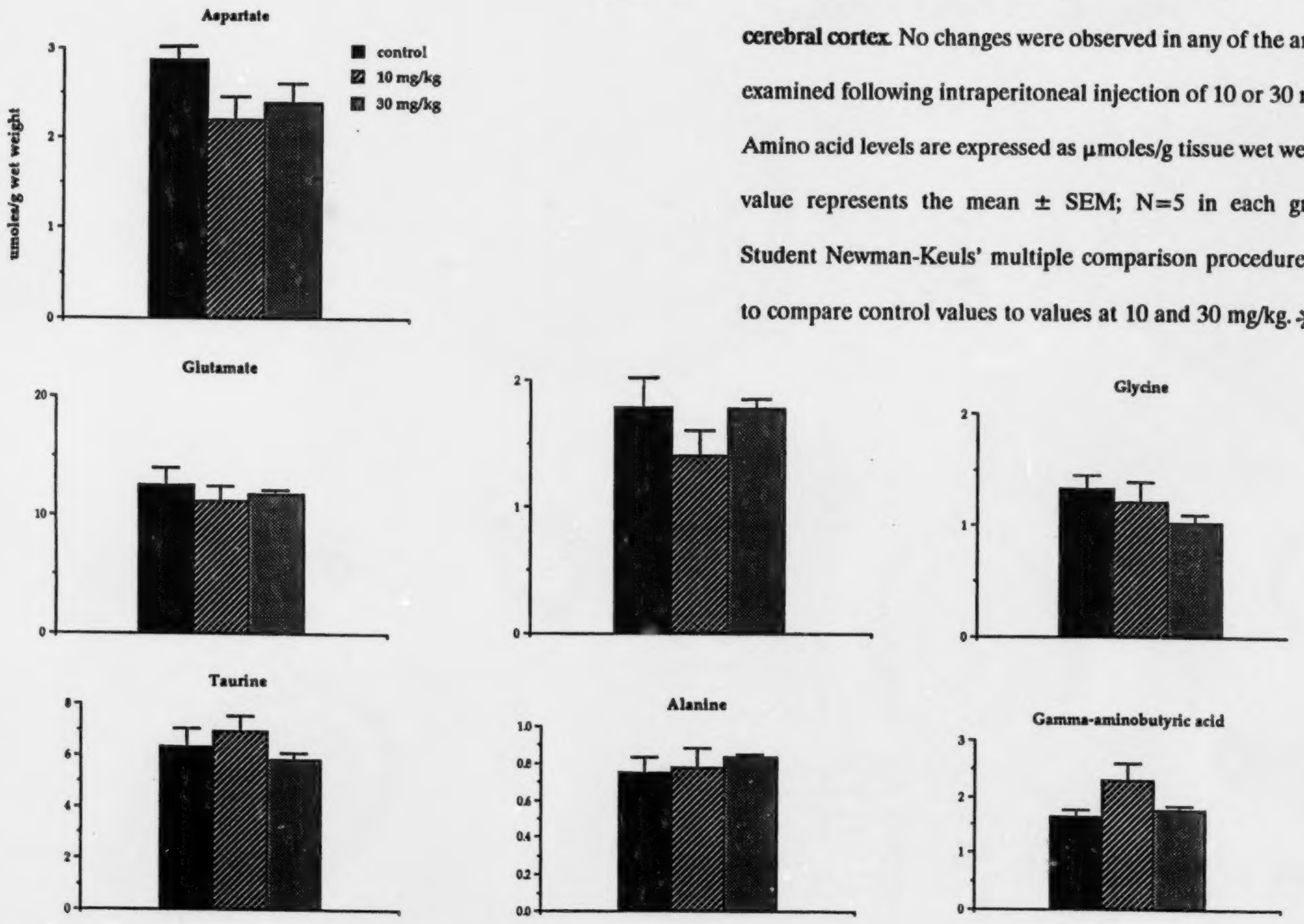
I. STUDIES ON TRANSMITTER LEVELS IN THE BRAIN.

The synthesis and storage of the neurochemicals that participate in the process of neurotransmission are important components of information processing and transfer in the central nervous system and perturbation of these processes can lead to inappropriate or ineffective communication between neurons. To examine whether sulfide toxicity results from an interference with transmitter synthesis and storage, we studied the effect of sulfide on amino acid and biogenic amine transmitter levels in brain regions that are known to control the neurological processes that show disturbance following sulfide poisoning. The regions investigated included the brain stem, hippocampus, striatum (caudate-putamen), cerebellum and cortex.

a. Effect of Sulfide on the Levels of Amino Acid Neurotransmitters.

Both excitatory and inhibitory amino acids are currently regarded as conventional neurotransmitters in several brain areas and synapses (Toleikis et al., 1978; Watkins and Evans, 1981). The study described in this thesis was done to test the hypothesis that sulfide would alter the levels of these amino acid transmitters. The substances examined included glutamate (GLU), aspartate (ASP) [both excitatory amino acid neurotransmitters (Curtis et al., 1959; reviewed by Watkins and Evans, 1981)]; glycine (GLY) and γ-aminobutyric acid (GABA) [both inhibitory amino acid neurotransmitters (Krnjevic, 1974 & 1987; Ottersen and Storm-Mathisen, 1984; Ribak, 1978; McCormick, 1989)]. Other amino acids examined were taurine (TAU), alanine (ALA) and serine (SER) [all inhibitory amino acids], and glutamine

FIGURE 9. Effect of HS⁻ treatment on total amino acid levels in the rat **cerebral cortex**. No changes were observed in any of the amino acids examined following intraperitoneal injection of 10 or 30 mg/kg HS⁻. Amino acid levels are expressed as µmoles/g tissue wet weight. Each value represents the mean ± SEM; N=5 in each group. The Student Newman-Keuls' multiple comparison procedure was used to compare control values to values at 10 and 30 mg/kg. *p< 0.05.



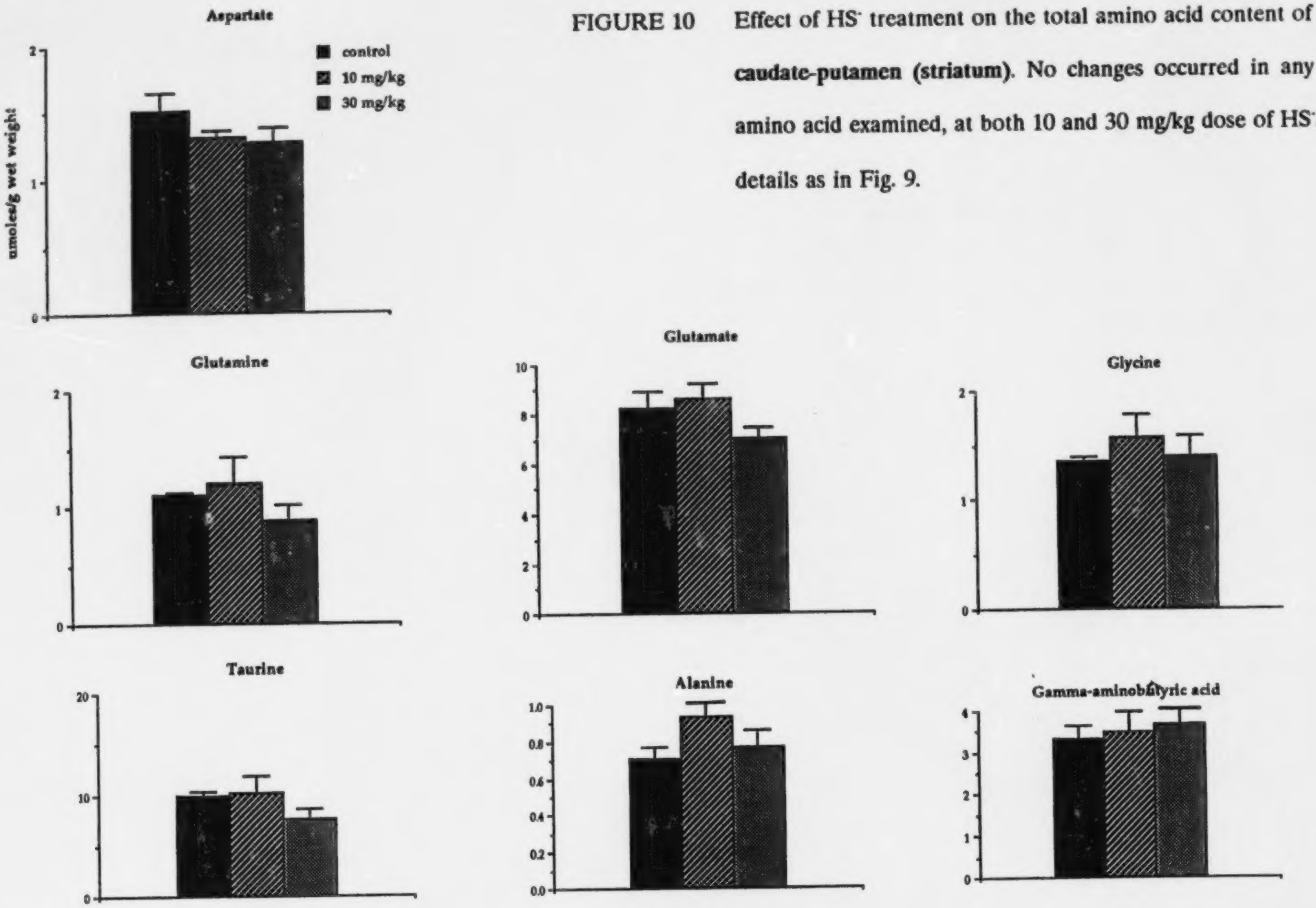


FIGURE 10 Effect of HS^- treatment on the total amino acid content of the rat **caudate-putamen (striatum)**. No changes occurred in any of the amino acid examined, at both 10 and 30 mg/kg dose of HS^-. Other details as in Fig. 9.

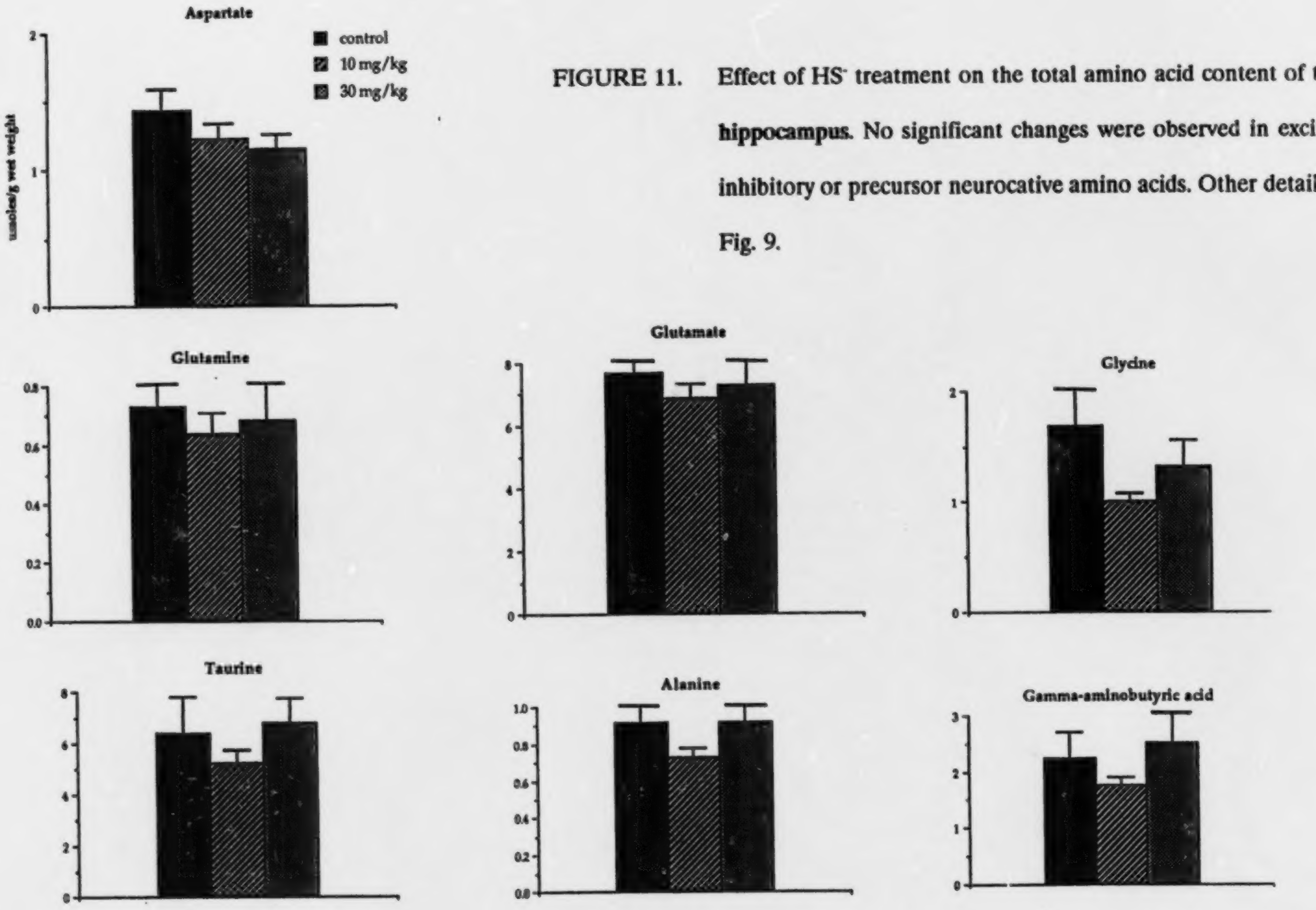


FIGURE 11. Effect of HS$^-$ treatment on the total amino acid content of the rat **hippocampus**. No significant changes were observed in excitatory, inhibitory or precursor neurocative amino acids. Other details as in Fig. 9.

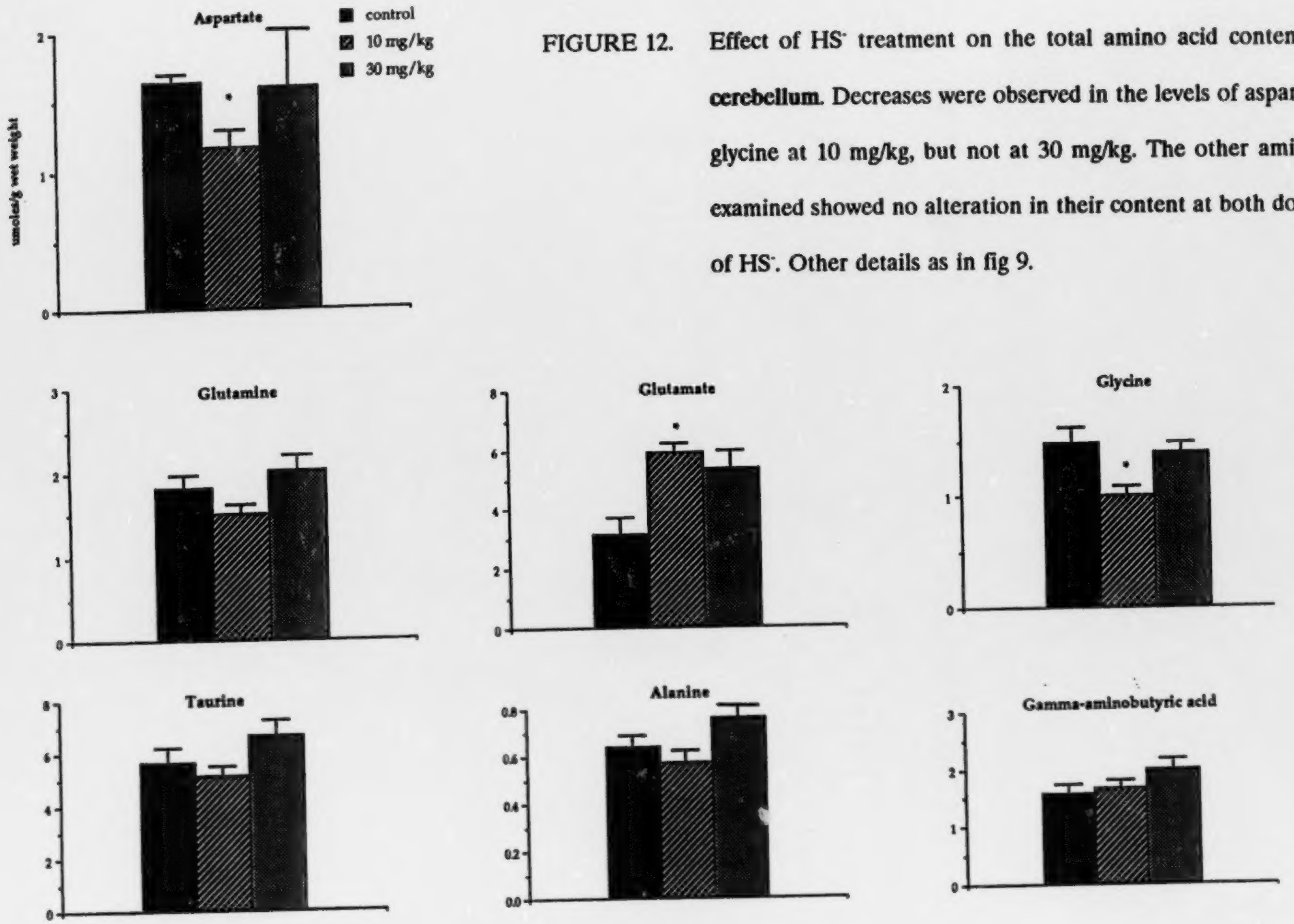


FIGURE 12. Effect of HS⁻ treatment on the total amino acid content of the **cerebellum.** Decreases were observed in the levels of aspartate and glycine at 10 mg/kg, but not at 30 mg/kg. The other amino acids examined showed no alteration in their content at both dose levels of HS⁻. Other details as in fig 9.

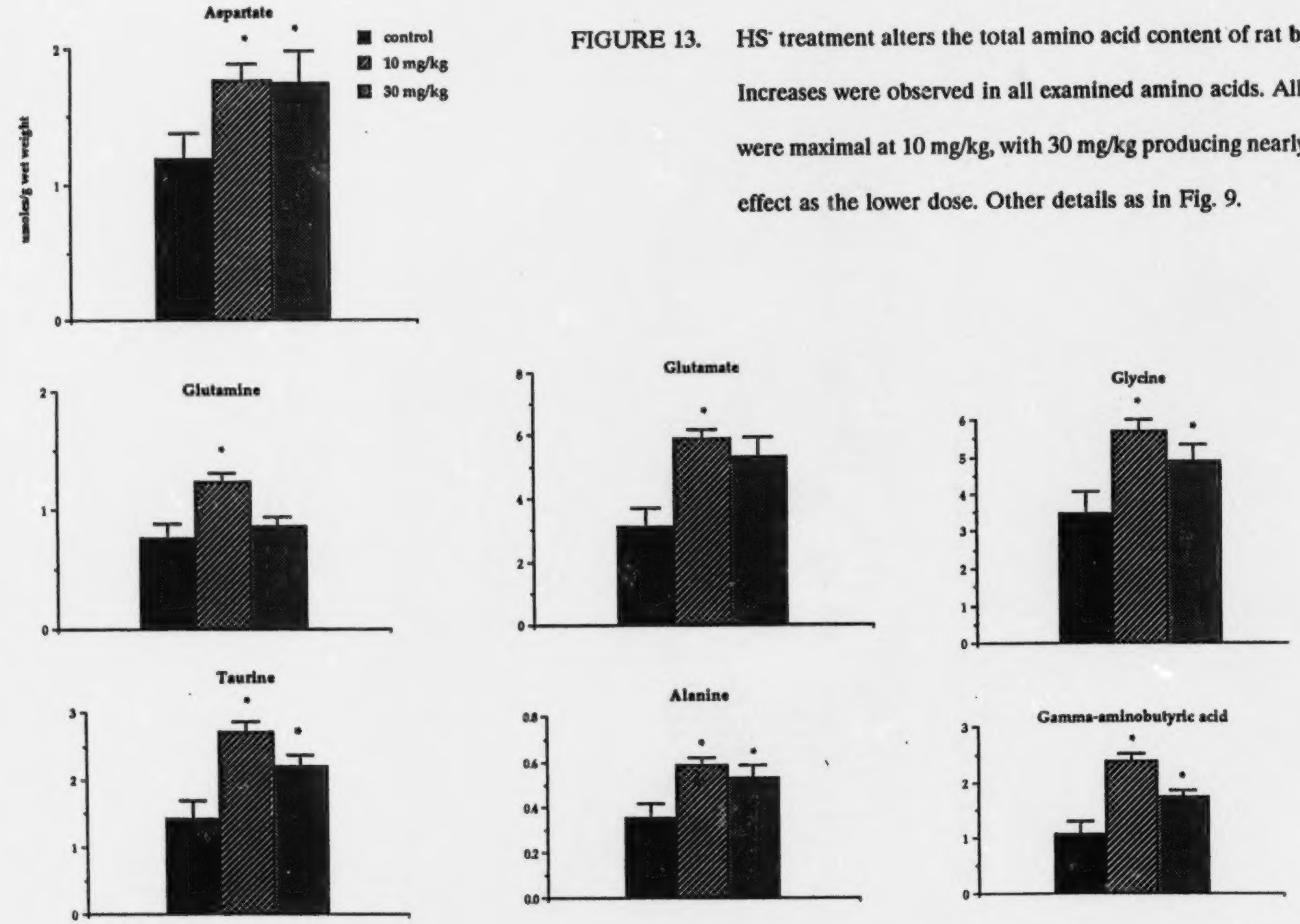


FIGURE 13. HS^- treatment alters the total amino acid content of rat brain stem. Increases were observed in all examined amino acids. All increases were maximal at 10 mg/kg, with 30 mg/kg producing nearly the same effect as the lower dose. Other details as in Fig. 9.

(GLN) (a precursor both of GLU and GABA). Although TAU, ALA and SER are neuroactive, there is little information about their role in normal physiological function of the CNS.

Comparison of amino acid levels in the above brain regions of rats treated by IP injection of different concentrations of NaHS (10 mg/kg and 30 mg/kg; LD_{50} = 15 mg/kg) to those in control animals (received IP injection of equivalent volume of saline) revealed no changes in any of the amino acids examined in the cerebral cortex, striatum or hippocampus ($p > 0.5$, $n=5$; Figs. 9, 10 & 11). The cerebellum showed a decrease of 30% from control values in the levels of both ASP and GLY at the dose of 10 mg/kg NaHS (Fig. 12), but not at the 30 mg/kg dose. No changes were seen in the other amino acids in this region. The brain stem was the region that showed the most change, with marked increases in levels of all amino acids at both doses of NaHS (Fig. 13). These increases peaked at a dose of 10 mg/kg as there was no statistical difference between 10 and 30 mg/kg of NaHS. The levels at the lower dose (10 mg/kg) were: ASP, 147.9%; GLU, 188.2%; GLN, 159.7%; GLY, 163.8%; TAU, 189.5%; ALA, 163.9% and GABA, 221.3% of their control levels.

b. Effect of Sulfide on the Synthesis and Storage of Biogenic Amines.

The catecholamines and 5-hydroxytryptamine (5-HT) have been implicated in the control and/or modulation of several brain processes. In particular, they are known to affect respiratory rhythm (Mueller et al., 1982; Murakoshi et al., 1985), motor activity (dopamine in the basal ganglia; Pijnenburg et al., 1975 and 1976;

FIGURE 14. HS^- treatment has no effect on catecholamine levels in the cerebral cortex and cerebellum. A. The cerebral cortex showed no change in the levels of any of the amines examined at both high and low doses of HS^-. B. In the cerebellum, only dopamine levels showed a significant increase in content following HS^- treatment at the higher dose. All values are expressed as the mean ± SEM in µg/g wet weight; N=5 in each group. Control animals received only saline. Statistical significance was taken at $p< 0.05$ using the Duncan's Multiple Range Test for comparison of control values to those at 10 and 30 mg/kg.

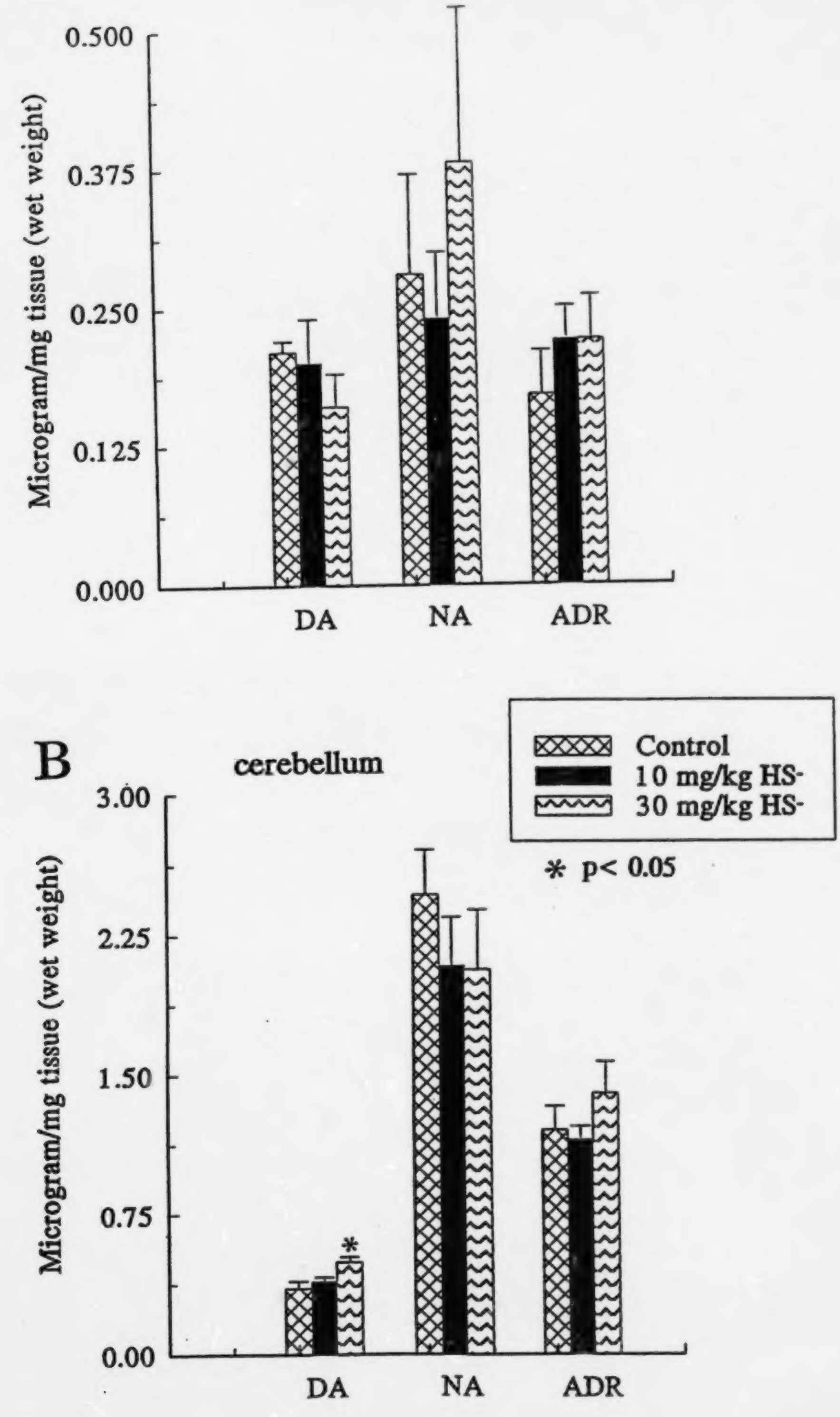
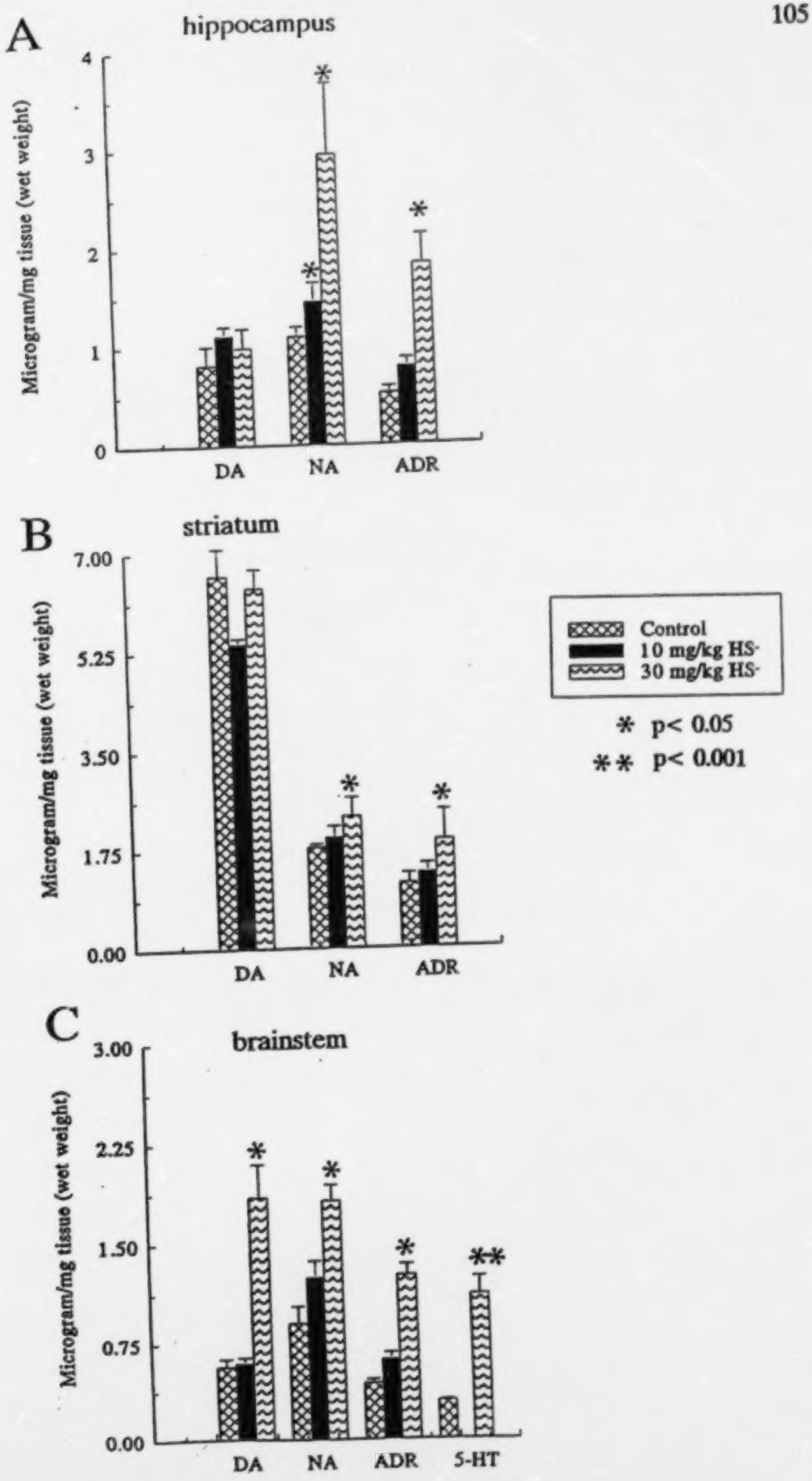

A
cortex
Microgram/mg tissue (wet weight)
0.500
0.375
0.250
0.125
0.000
DA
NA
ADR
B
cerebellum
Microgram/mg tissue (wet weight)
3.00
2.25
1.50
0.75
0.00
DA
NA
ADR
Control
10 mg/kg HS-
30 mg/kg HS-
*
* p< 0.05

FIGURE 15. HS^- treatment leads to increases in biogenic amine levels in the hippocampus, striatum and brain stem. A. Significant elevation in noradrenaline (NA) and adrenaline (ADR) levels were observed in the hippocampus following 30 mg/kg HS^- as compared to control. NA also showed increases at the lower dose of 10 mg/kg. B. The striatum showed increases in NA and ADR, but not dopamine (DA) which is abundant in this nucleus. These changes occurred only at the higher dose. C. The brain stem showed increases in all catecholamines and also in 5-hydroxytryptamine (5-HT), but only at the higher dose of 30 mg/kg. Other details as in fig 6.


A
hippocampus
Microgram/mg tissue (wet weight)
0
1
2
3
4
DA
NA
ADR
*
*
*
B
striatum
Microgram/mg tissue (wet weight)
0.00
1.75
3.50
5.25
7.00
DA
NA
ADR
*
*
▨ Control
■ 10 mg/kg HS-
▨ 30 mg/kg HS-
* p< 0.05
** p< 0.001
C
brainstem
Microgram/mg tissue (wet weight)
0.00
0.75
1.50
2.25
3.00
DA
NA
ADR
5-HT
*
*
*
**

Pijnenburg and van Rossum, 1973) and probably memory processes (Madison and Nicoll, 1982; Bliss et al., 1983). Since the clinical symptoms following sulfide intoxication include alterations in respiration and deficits in motor co-ordination and memory, we examined whether sulfide produced these effects by altering the normal turnover of these substances. This study was conducted on brain regions implicated in the above processes, including the brain stem, hippocampus, striatum, cerebellum and cortex.

Following IP injections of 10 and 30 mg/kg NaHS, of the five regions studied, the cortex showed no changes in all catecholamines and 5-HT levels (Fig. 14A). In the cerebellum, only dopamine increased (to 140 ± 6.1% of control levels; $p < 0.05$; $n=5$) at the 30 mg/kg dose, but not at the lower dose (Fig. 14B). In the hippocampus, levels of noradrenaline and adrenaline increased to 268 ± 24.8% and 356 ± 16.5% respectively, of control levels ($p < 0.05$; $n=5$) at 30 mg/kg. At the 10 mg/kg dose, there was a significant increase in noradrenaline only (to 133 ± 13% of its control levels; $p < 0.05$; $n=5$). Dopamine levels did not show any changes in this region (Fig. 15A). In the striatum (caudate-putamen), noradrenaline and adrenaline levels were significantly elevated at 30 mg/kg, to 131 ± 14.2% and 171 ± 27.5% respectively, of their control levels ($p < 0.05$, $n=5$; Fig. 15B). No changes were observed in the levels of dopamine at 30 mg/kg and all biogenic amines at the lower dose of 10 mg/kg NaHS ($p > 0.05$; $n=5$; Fig. 15B). In the brain stem, large increases were observed in levels of all the catecholamines examined after 30 mg/kg NaHS treatment. Noradrenaline increased to 202 ± 6.6%, adrenaline to 293 ± 6.4% and dopamine to

325 ± 13.5% of their control levels ($p < 0.05$; n=5; Fig. 15C). Levels of 5-HT were elevated to 383 ± 11.7% of control levels ($p < 0.01$; n=5; Fig. 15C) in the brain stem of rats exposed to 30 mg/kg NaHS.

II. TRANSMITTER RELEASE STUDIES.

Synaptic transmission involves the spontaneous or evoked release from presynaptic terminals of transmitters which then interact with postsynaptic receptors to generate a characteristic response (see introduction). The magnitude of the postsynaptic response is determined, in part, by the amount of transmitter released and how rapidly it is metabolised or removed from the synapse. The magnitude of the response determines the success of the information transfer from pre- to postsynaptic neurons. Alteration in either the presynaptic release process or the metabolism and/or removal after release can profoundly affect the efficiency of synaptic transmission. Inappropriate alteration can therefore lead to aberrant synaptic transmission and hence CNS malfunction. To determine whether sulfide produced its toxicity by perturbation of this aspect of neurotransmission, we studied the effects of HS^- on the release of neuroactive amino acids from selected brain regions, including the hippocampus, the striatum and the brain stem *in vivo*. This was achieved by recovering perfusates from the selected brain regions *in vivo*, using the concentric cannula, push-pull perfusion technique (Gaddum, 1966; Hudson et al., 1986; Roisin et al., 1991). This technique was shown by Yaksh and Yamamura (1974) to closely reflect on-going changes in local concentrations of brain substances without gross cellular damage.

Because of the known sources of variability inherent in this technique (Yaksh and Yamamura, 1974), the determination of absolute amounts of transmitters was considered inaccurate. At the beginning of each experiment, efforts were made to minimize these effects. Each animal was perfused for at least 30 min, to equilibrate and stabilize the recovery process, before samples were taken. For this reason, the experimental protocol was to obtain 2 or 3 ten minute control samples following the equilibration period (only samples later verified not to differ from each other significantly were included in the analysis), and then the collection of 3-4 more 10 minute samples post HS^- treatment. Because the CNS has efficient uptake mechanisms for GABA and GLY in particular (e.g. neuronal and glial; Iversen and Neal, 1968), the collecting efficiency of these amino acids in the perfusate was enhanced by the inclusion of a GABA uptake inhibitor, nipecotic acid (5 μM) in all perfusion solutions. In the analyses, the average of the absolute amounts in the control samples was used to normalise the subsequent samples to control (see Methods).

a. Effect of Sulfide on the Release of Amino Acids in the Striatum (Caudate-Putamen)

Amino acids have been shown to be important neurotransmitters in the CNS (reviewed by Collingridge and Lester, 1989), and their involvement in the co-ordination of motor activity has been demonstrated (Stone, 1979; Herrling, 1985).

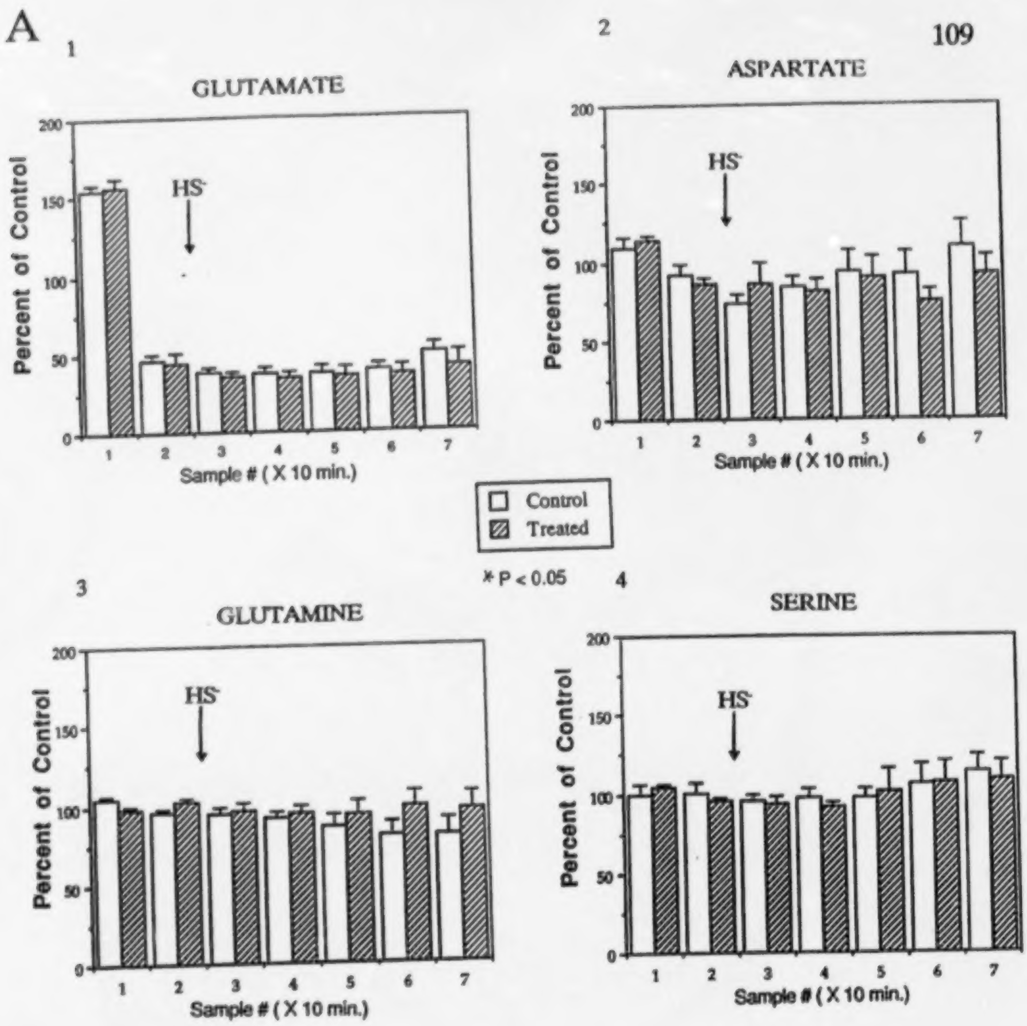
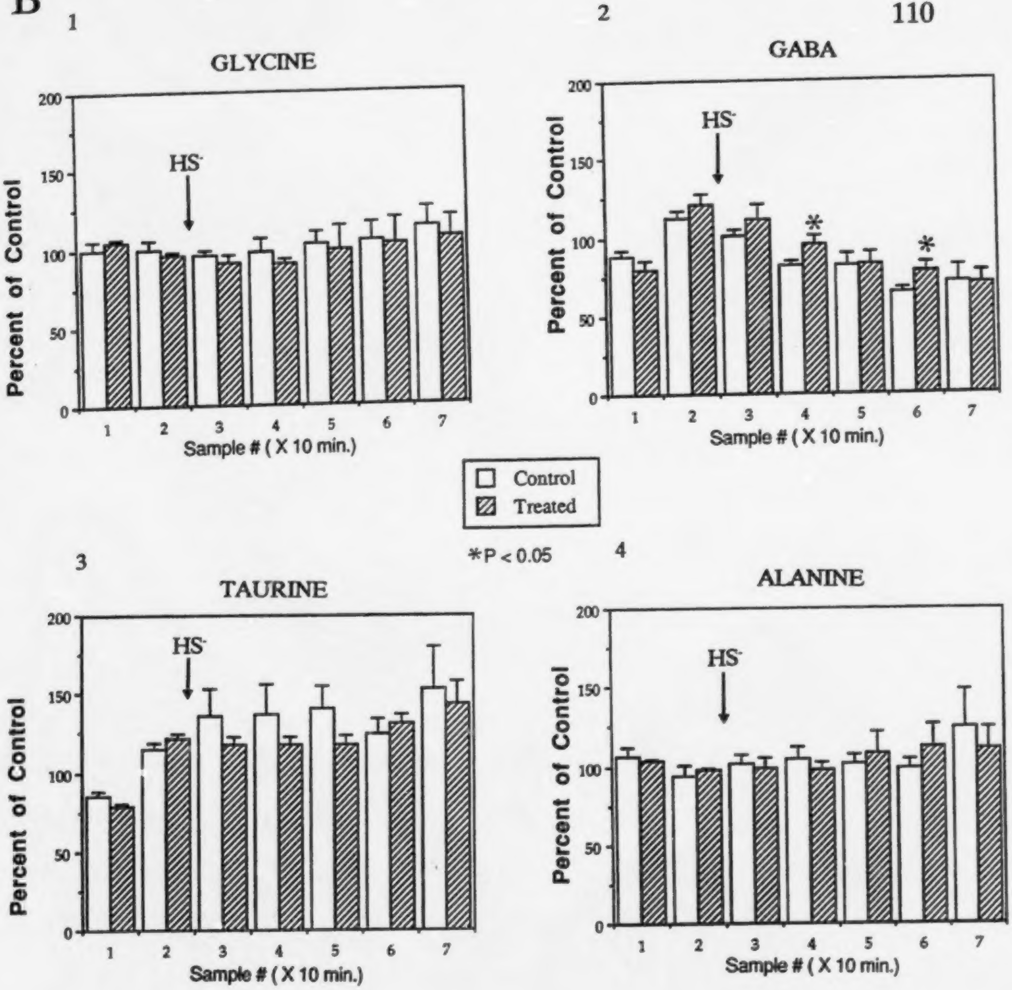


FIGURE 16 Effect of intraperitoneal injection of HS^- on the release of neuroactive amino acids in the **caudate-putamen (striatum)**. A. IP injection of 15 mg/kg does not alter the release pattern of excitatory amino acids in the striatum when compared to their time-matched controls. Continued next page.

B



B. A sustained increase in the release of GABA was detected following this treatment as compared to their time-matched control. No changes were seen in the release of the other inhibitory neuroactive amino acids. N=8 in both groups. Asteriks mean statistical significance compared with their time-matched control by unpaired student t-test.

Following sulfide poisoning both in humans and in rats, victims demonstrate severe changes in motor activity (see introduction). We were interested in knowing if sulfide produced these effects through alteration in the release and/or metabolism of excitatory and/or inhibitory amino acid neurotransmitters. Such an action will result in changes in the amount of retrievable transmitter in the immediate extracellular environment (Bayon and Drucker-Colin, 1985; Roisin et al., 1991). We therefore tested the hypothesis that sulfide will alter the amount of amino acids recovered from the selected brain regions. The amounts of the following amino acids were examined following NaHS treatment: GLU, ASP, GLN, TAU, ALA, GABA, SER and GLY. All amounts recovered following sulfide treatment were compared with their time-matched control levels by the unpaired Student's t-test. Control animals received equivalent volumes of saline by the same route.

IP injection of 15 mg/kg (LD_{50}) of NaHS increased the amount of recovered GABA to 120 ± 5.5% of control levels ($p < 0.05$, $n=8$). No significant changes were seen in the recovered amounts of the other amino acids studied (Fig. 16A & B).

It has been pointed out earlier that for toxic manifestations to occur with HS^-, the magnitude of the exposure must overwhelm the removal and degradatory processes (see Introduction). Since these processes may be altered under anaesthesia, and the striatum is a structure that is known to rapidly degrade or less selectively accumulate sulfide compared to other brain regions (Warenycia et al., 1989), we examined if direct application of sulfide to striatal neurons, via the perfusion fluid, would alter the amount of amino acid transmitter available for retrieval. Based on the

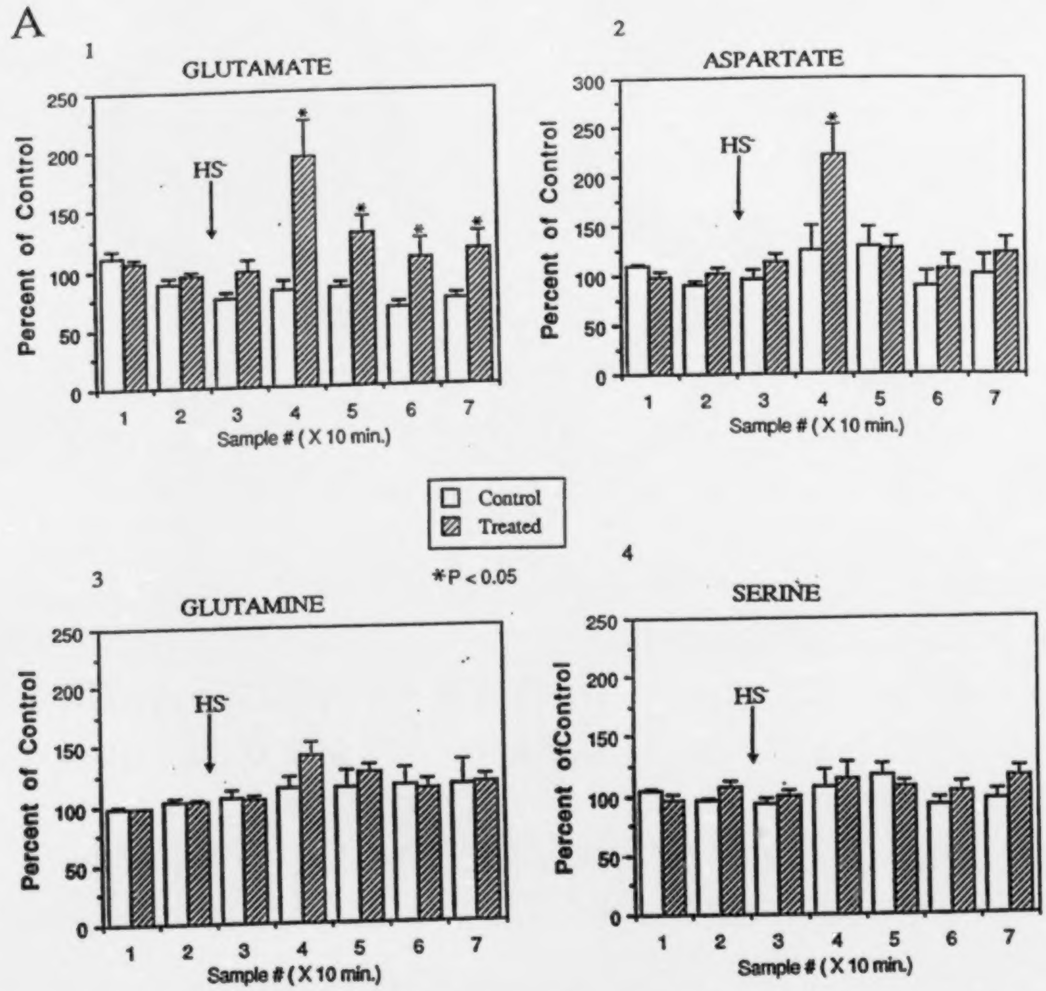
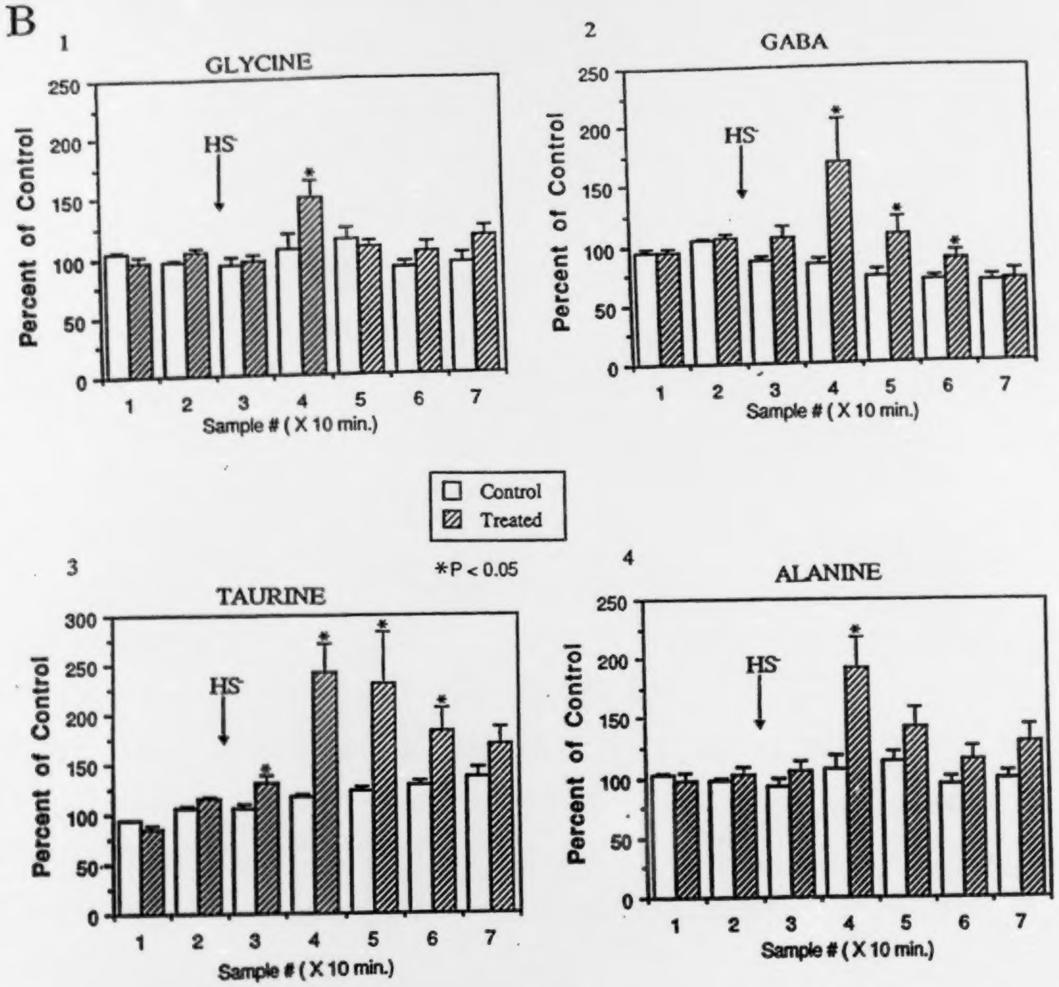


FIGURE 17. Direct perfusion of the **caudate-putamen (striatum)** with a solution of HS^- altered the levels of both excitatory and inhibitory amino acids in the recovered perfusate. A. 10 min perfusion (at a rate of 20 μl/min) with 3 μg/ml solution of HS^- caused a sustained increase in the release of glutamate, while producing only a transient increase in aspartate release. No changes in release were seen with glutamine and serine. Continued next page.



B. Transient increases in the release of glycine and alanine were detected, whereas sustained increases were seen with GABA and taurine release, all compared to their time-matched controls. N=9 in both groups, other details as in Fig. 16.

observation by Warenycia et al. (1989) that following IP injection of sulfide only a fraction reached the brain, we selected a concentration that reflected the amount of sulfide reaching the brain. When the LD_{50} dose of HS^- (15 mg/kg) was given by ip injection, approximately 3 μg/g tissue wet weight was detected in the brain. We therefore applied this concentration of sulfide directly into the striatum for 10 min. Perfusion for this time interval with a solution of 3 μg/ml sulfide (dissolved in ACSF) caused an initial increase (first 10 min sample) in the amount of recovered GLU to 236 ± 29% of control level ($p < 0.05$, $n=9$). This amount then declined to 154 ± 15% of control values ($p < 0.05$; Fig. 17A1) in the following 10 min sample and remained at the latter level in subsequent samples. A transient increase in the amount of ASP to 177 ± 31% of control ($p < 0.05$; Fig. 17A2) was also observed. No changes were observed in the amounts of GLN and SER recovered (Fig. 17A). Analysis of the inhibitory amino acids showed persistent increases in GABA. The first sample after NaHS treatment increased to 203 ± 36% of its time-matched control value ($p < 0.05$; Fig. 17B2). Subsequent samples showed a gradual decline in magnitude but was still significantly higher than the time-matched control levels. Taurine levels increased to 123 ± 7.9% of control levels immediately following sulfide application. This value increased to 205 ± 30% of control levels in the second 10 min sample following sulfide treatment and remained close to this level for 20 min before returning to control levels ($p < 0.05$;Fig. 17B3). GLY and ALA showed only brief increases in their amounts, to 142 ± 14.9% and 178 ± 25% respectively of their time-matched controls (Fig. 17B1&4; $p < 0.05$).

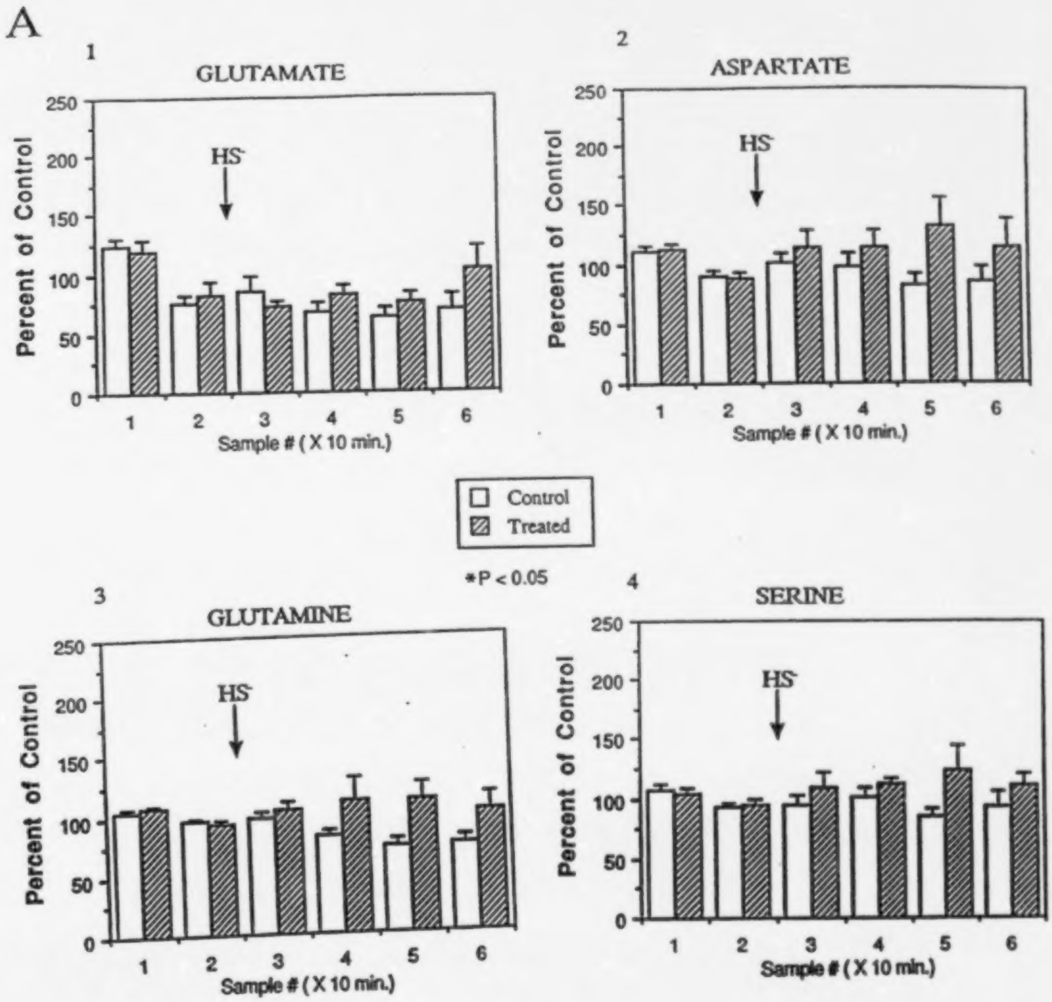
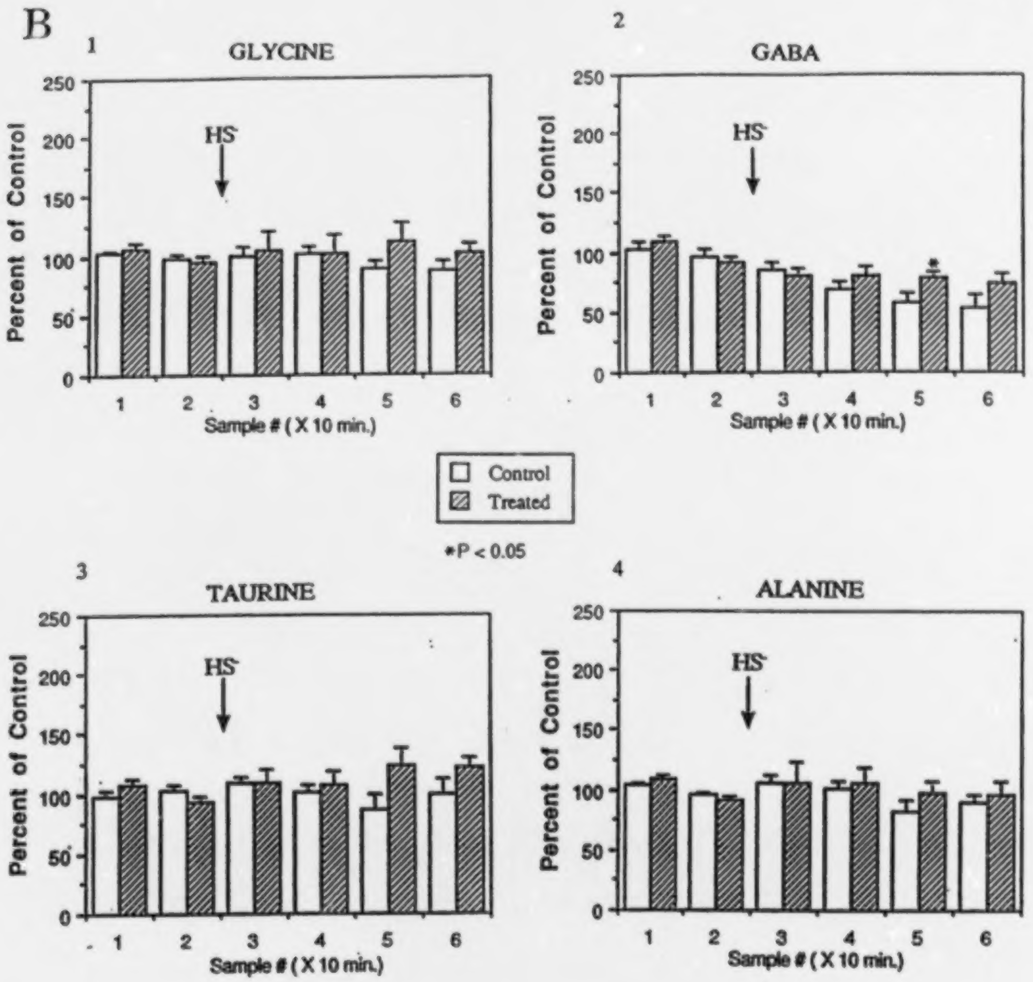


FIGURE 18. Intraperitoneal injection of HS⁻ does not alter the release pattern of amino acid transmitters in the **hippocampal area CA1** following slow, low intensity stimulation of commissural fibers. A. Neither aspartate nor glutamate or its precursor, glutamine showed any detectable changes in their release when compared to their time-matched controls. Also no change was seen in the release of the excitatory neuroactive amino acid. Continued next page.



B. Only a delayed increase was detected in the amount of GABA in recovered perfusate (135 ± 5.4%). No changes were detected in glycine and the other inhibitory neuroactive amino acids (taurine and alanine) when compared to their time-matched control. N=8 in both groups, other details as in Fig. 16.

b. Effect of Sulfide on the Release of Amino Acids in the Hippocampus.

Because survivors of exposure to high levels of hydrogen sulfide gas often display retrograde amnesia and because the hippocampus has been implicated in memory, for example place memory in rats (O'Keefe and Nadel, 1978) and a complex amnesic syndrome in humans (O'Keefe and Nadel, 1978; Corsi, 1972; Milner, 1968), we examined whether sulfide produced this effect by altering the release of amino acid neurotransmitters in the area CA1 of the hippocampus. To test the hypothesis that sulfide altered transmitter release or metabolism in CA1 and, presumably via such an action, altered memory processing, storage and retrieval, two routes of sulfide application were employed: IP injection of a solution of NaHS 15 mg/kg (LD_{50}) and 10 min perfusion with a 4 μg/ml (equivalent to amount of HS^- found in hippocampus after IP injection of LD_{50} concentration) solution of HS^- dissolved in ACSF. Sulfide-treated animals (n=8) were compared with their time-matched controls (saline-treated; n=8). Hudson et al. (1986) had shown earlier that the recovery of amino acids (basal release) in the hippocampus was very low unless release was evoked by stimulation (chemically with a high K^+ concentration or electrically via stimulation of afferents to the CA1 such as the perforant path of the hippocampal commissure). In this study, release was enhanced by slow electrical stimulation (1 Hz, 5-10 V) of the dorsal hippocampal commissure.

Following IP injection of 15 mg/kg NaHS (LD_{50}), only a delayed change in the amount of GABA recovered was observed. GABA levels increased to 135 ± 5.41% of their control levels ($p < 0.05$; Fig. 18B2). No changes were detected in the amounts

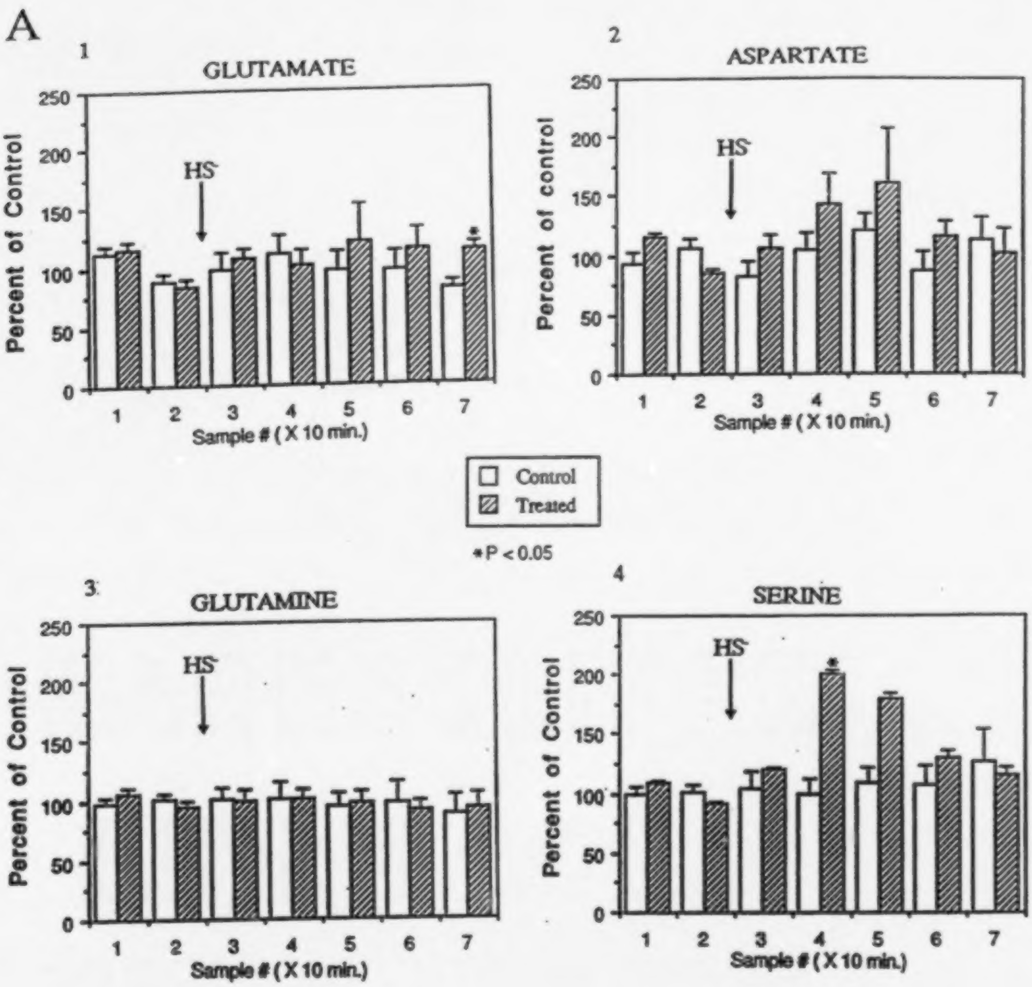
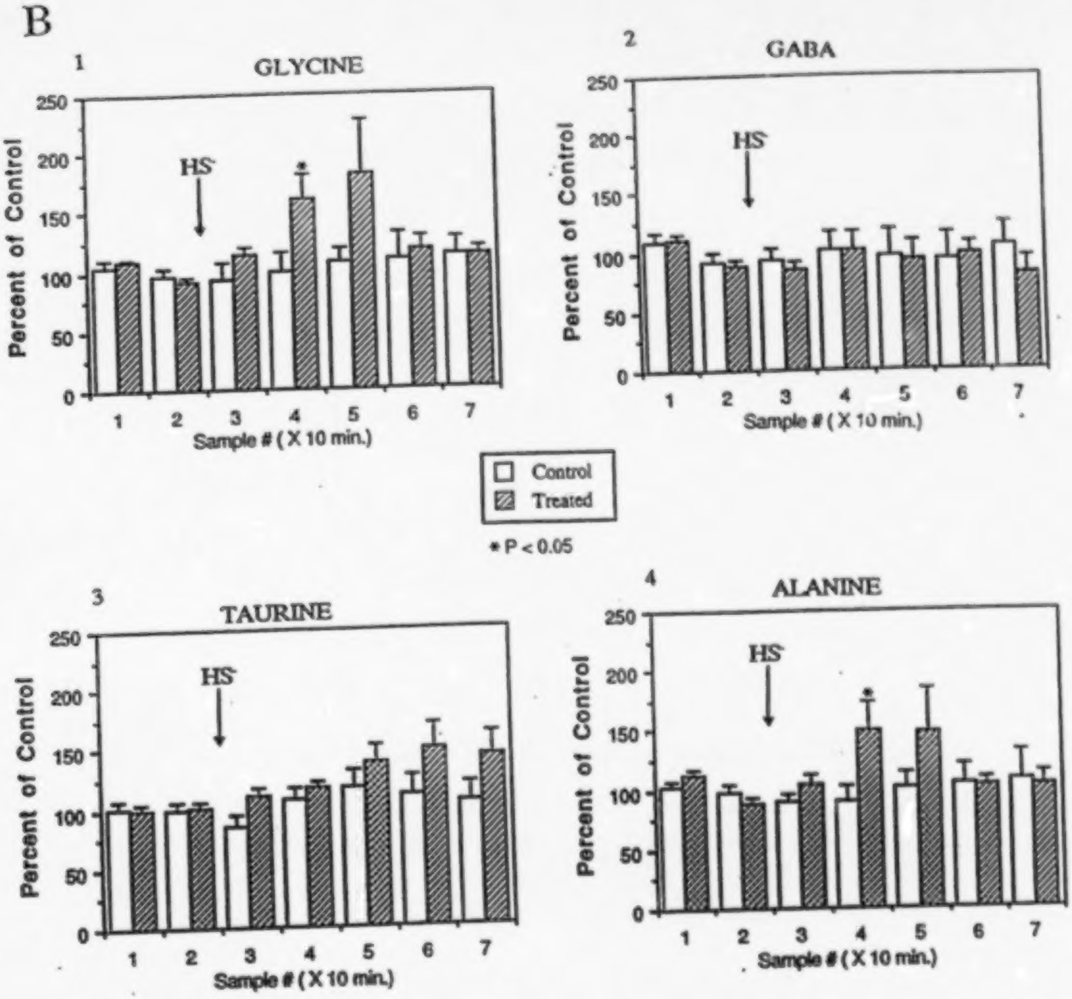


FIGURE 19. Effect of direct perfusion with HS^- of hippocampal area CA1 on evoked amino acid transmitter release following slow, low intensity stimulation of the commissural fibers. A. 10 min perfusion of the area CA1 with a solution of ACSF containing 4 µg/ml HS^- caused a delayed increase in the amount of glutamate in recovered perfusate, but not its precursor, glutamine. Aspartate levels increased compared to their time-matched controls but the large standard error made this increase statistically insignificant. Serine also showed an early but transient increase in release as compared to its time-matched control. Continued next page.



B. Transient increases in the release of glycine, and the neuroactive inhibitory amino acid alanine were detected. No changes were seen in the release of GABA and taurine. N=7 in both groups, other details as in Fig. 16.

of the other amino acids (Fig. 18A & B). Ten minute perfusion of the CA1 area with a 4 μg/ml solution of HS^- in ACSF caused increases in the amount of some amino acids recovered from this region. A delayed increase in GLU to 202 ± 34.4% of its control levels was observed (Fig. 19A1), while a rapid and sustained increase occurred in recovered SER levels (Fig. 19A4). Also, rapid increases in the amounts of GLY (161.6 ± 19.5%) and ALA (165.2 ± 23.3%) were detected which returned to their control levels in 20 min (Fig. 19B1 & 4 respectively). No significant changes in the amounts of the other amino acids were detected.

c. Effects of Sulfide on the Recovery of Amino Acids from a Brain Stem Reticular Nucleus.

Several nuclei in this region of the brain stem have been implicated in the generation or maintenance of respiratory rhythms. The nucleus ambiguus and retroambiguus have particularly been shown to be involved in generation or regulation of respiratory rhythm (Bystrzycka and Nail, 1985). Also, the areas bordering this region have been shown to contain clusters of cells that control cardiovascular and respiratory processes (Beger et al., 1977; Feldman et al., 1977; Feldman et al., 1981; Bystrzycka and Nail, 1985). As respiratory control involves precise coupling of several neural processes, perturbation of neural signalling will be expected to lead to respiratory arrest. If HS^- were to sufficiently perturb the release and/or metabolism of transmitters (after release) involved in this coupling, then respiratory arrest would result. Such a perturbation should be reflected in the amount

A

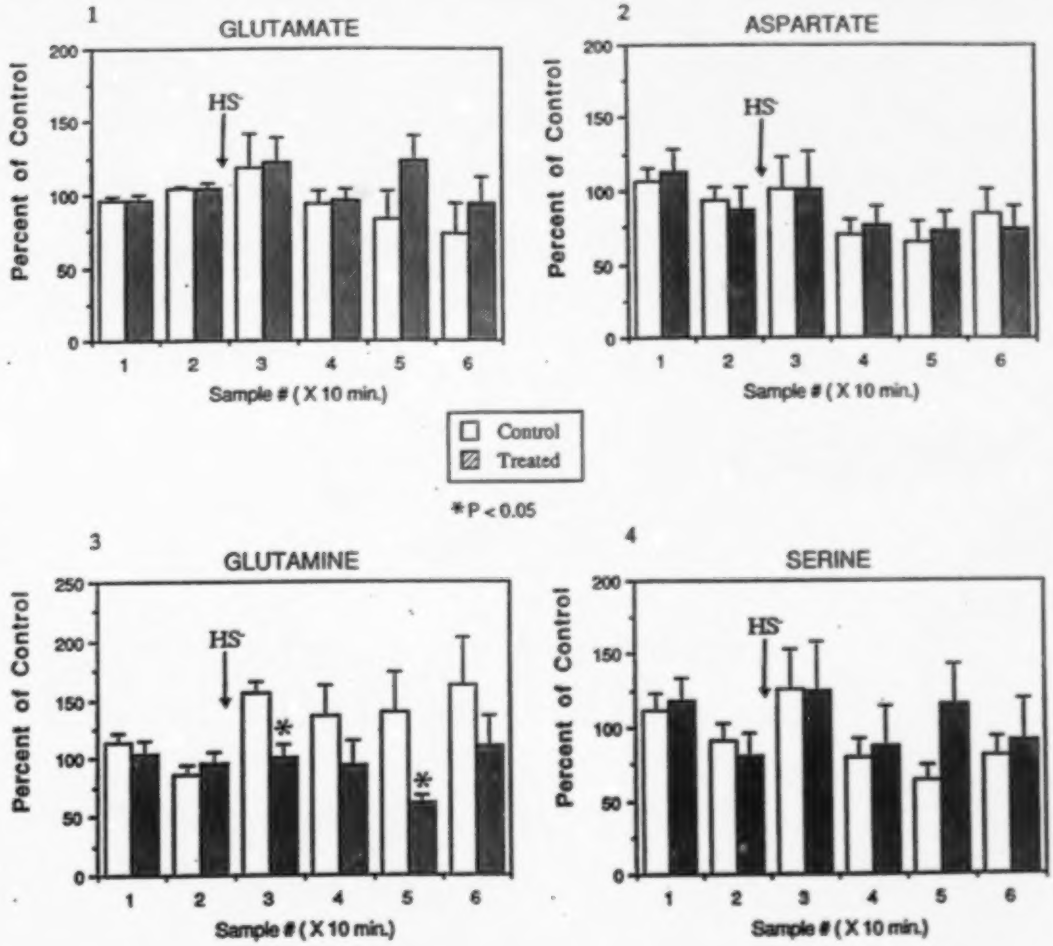
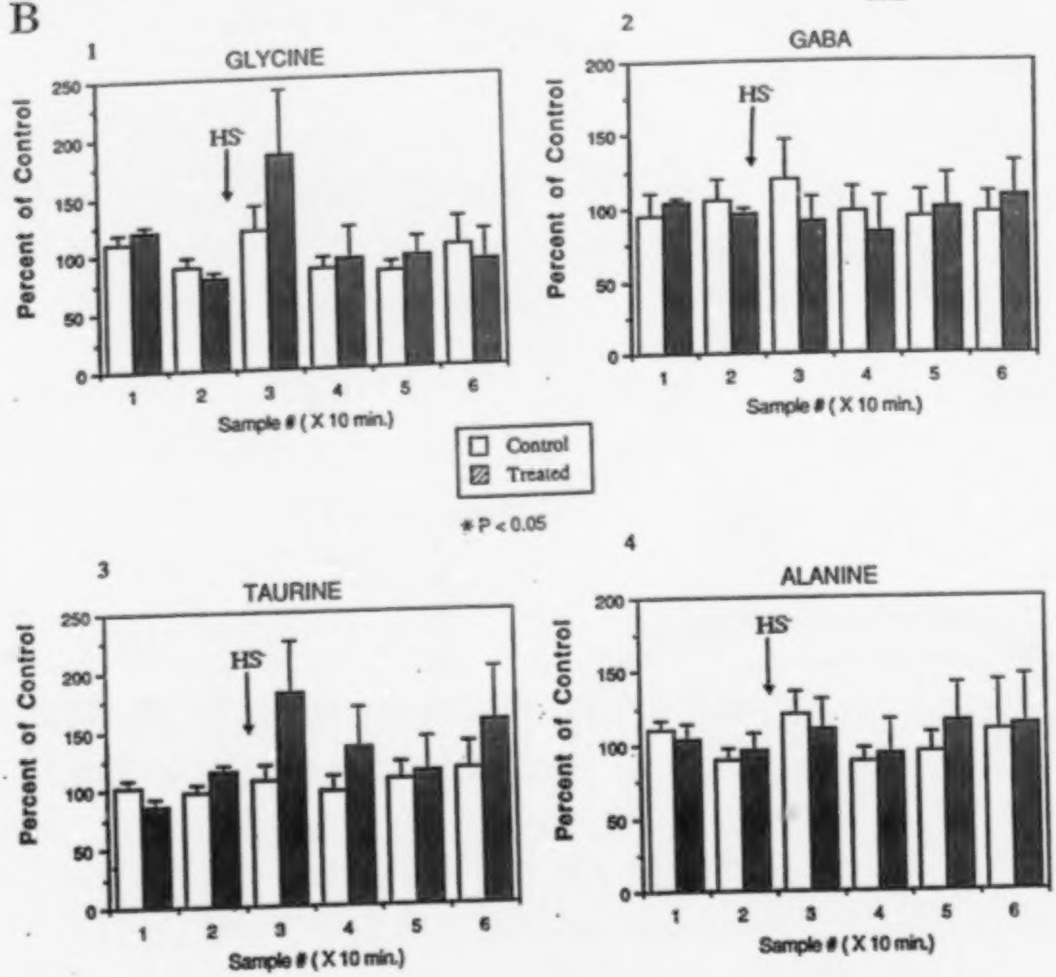


FIGURE 20. HS⁻ does not alter the levels of amino acid neurotransmitters recovered from a **brain stem reticular nucleus.** A. Intraperitoneal injection of 15 mg/kg HS⁻ caused a decrease in the release of only glutamine (a precursor of glutamate and GABA), but not glutamate or aspartate, the putative excitatory amino acid neurotransmitters. No change in release was seen for serine, an excitatory neuroactive amino acid. Continued next page.



B. No changes in amino acid recovery was seen in both putative inhibitory amino acid neurotransmitters (GABA and glycine) and in inhibitory neuroactive amino acids (taurine and alanine); n=5 in both groups. Other details as in Fig. 16.

of transmitters available for collection. To test the hypothesis that HS^- would alter the release or metabolism or both of transmitters in the brain stem, we recovered released amino acids in the area bordering and including the dorsal portion of the nucleus ambiguus in rats after exposure to HS^-. The release of both excitatory and inhibitory amino acids was examined in the above area as a representative respiratory nucleus. The amounts of GLU, ASP, GABA, GLY, TAU, GLN, SER and ALA in retrieved perfusates were examined at different sulfide concentrations and routes of administration. Normalised controls were compared with their time-matched normalised sulfide-treated samples.

Intraperitoneal injection of NaHS at the dose of 15 mg/kg (LD_{50}) caused no significant changes in the amounts of either excitatory (GLU, ASP and SER) or inhibitory (GABA and GLY, TAU, ALA) amino acids recovered (Fig. 20). However, a decrease in the amount of recovered GLN to 64.5 ± 11% ($p<0.05$) of control was observed in the first 10 min sample. A further decrease to 43.5 ± 6.9% of the control level was observed in the 20 min sample ($p< 0.05$, $n=5$; Fig. 20A3). These decreases were not accompanied by changes in GLU and/or GABA levels.

To determine whether direct application of HS^- would alter the amounts of amino acids recovered, two different concentrations of HS^- were applied via the perfusion fluid. Because the brain stem was shown by Warenycia et al. (1989) to selectively take up HS^- following poisoning and because this region had in previous neurochemical studies (this thesis) been found to be very sensitive to HS^-, we examined if direct, relatively short periods of application of low concentrations of HS^-

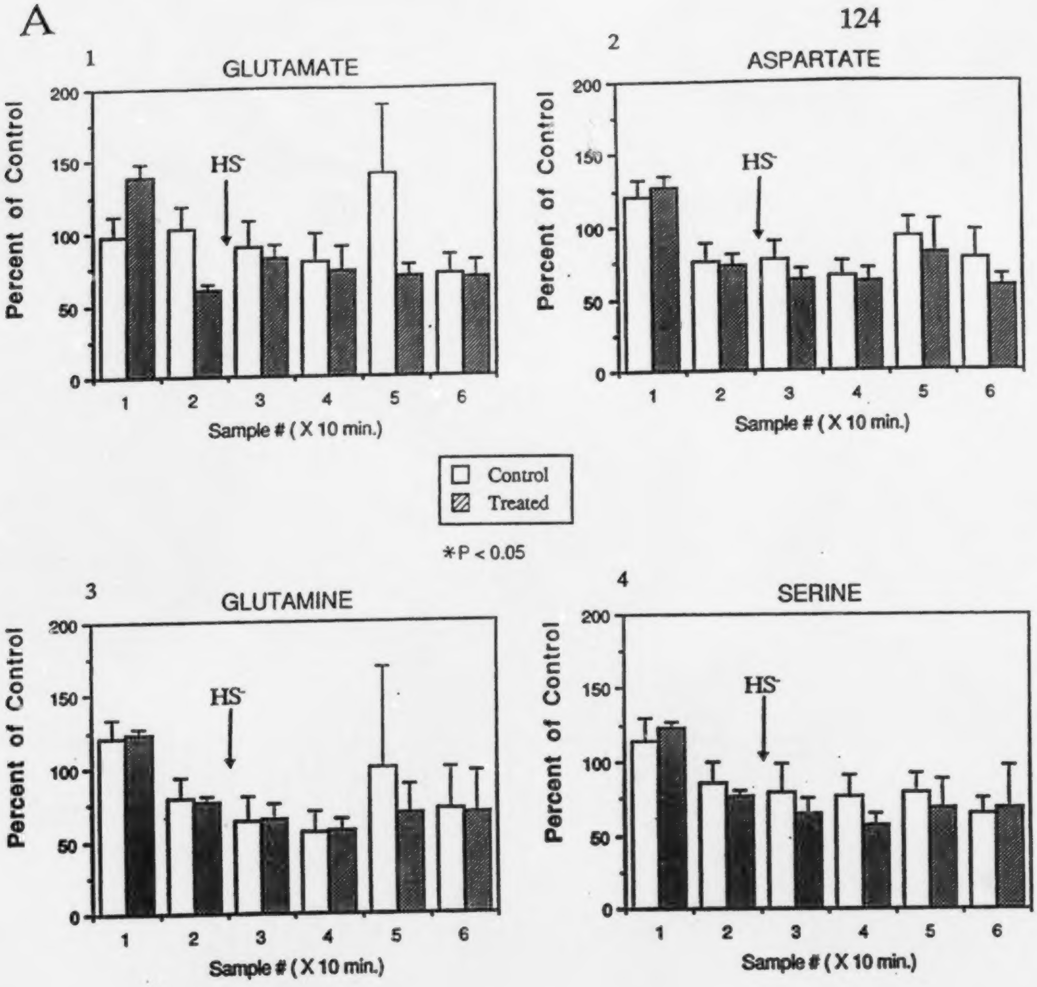
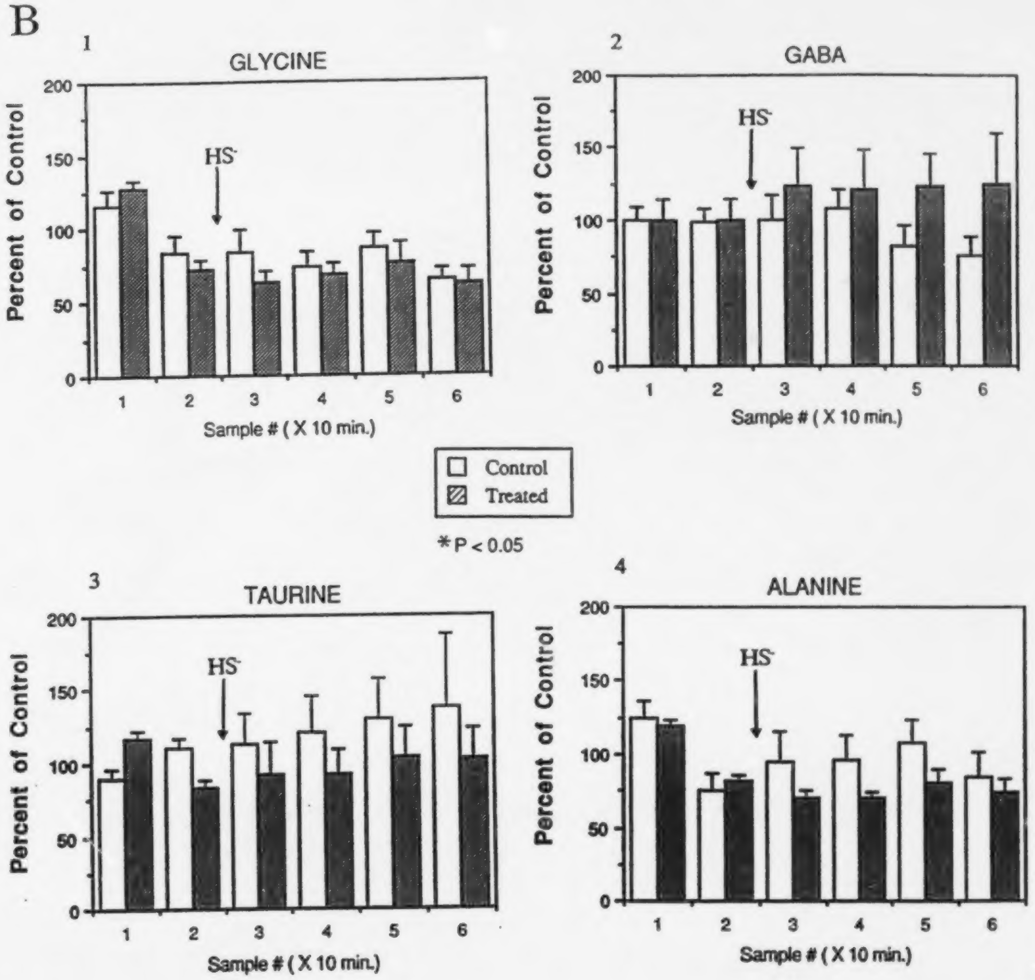


FIGURE 21. Effect of direct perfusion with a low concentration of HS⁻ on amino acid recovery from a rat **brain stem reticular nucleus.** The endogenous amount of HS⁻ in rat brain stem is approximately 1.2 μg/g tissue (Warenycia et al., 1987). Direct, 2 min perfusion (at the rate of 20 μg/ml) of rat brain stem reticular nucleus with a solution of 2 μg/ml HS⁻ (in ACSF) does not alter the amount of amino acids recovered from this nucleus. A & B. No changes were seen in either excitatory or inhibitory amino acids. N=6 in both groups, other details as in Fig. 16.


B
1
GLYCINE
2
GABA
3
TAURINE
4
ALANINE
Percent of Control
0
50
100
150
200
HS-
1
2
3
4
5
6
Sample # (X 10 min.)
☐ Control
▨ Treated
*P < 0.05

would induce noticeable changes in the release or metabolism of amino acid transmitters. By comparison to other regions, such as the hippocampus and striatum where higher doses of sulfide were applied over longer periods, several animals died during these experiments. Relatively fewer animals died with IP injection and the perfusion with 2 μg/ml in comparison to perfusion with 3 μg/ml solution of sulfide which caused significant mortality in rats, probably indicating that it was effective in inhibiting respiration. Only animals which survived to the end of experiments were including in the analyses.

Application of 2 μg/ml HS^- (marginally above the endogenous amount in brain stem) for 2 min by perfusion produced no measurable changes in the amount of amino acids recovered (Fig. 21A & B). However, 2 min perfusion with 3 μg/ml HS^- solution (close to the amount found in brain stem following IP injection of NaHS at the LD_{50} dose and significantly higher than the endogenous level) produced a delayed decrease to 56.1 ± 9.7% of control ($p < 0.05$; $n=6$; Fig. 22B1) only in the levels of GLY, an important inhibitory transmitter in the brain stem and spinal cord. No changes were observed in the levels of the other amino acids examined (Fig. 22 A & B). The delayed nature of the effect makes it unlikely to play a role in respiratory paralysis, as death usually occurs within 2 min of ip injection of NaHS. Thus, it may represent only an alteration that may contribute to the neurological sequelae in survivors of sulfide poisoning.

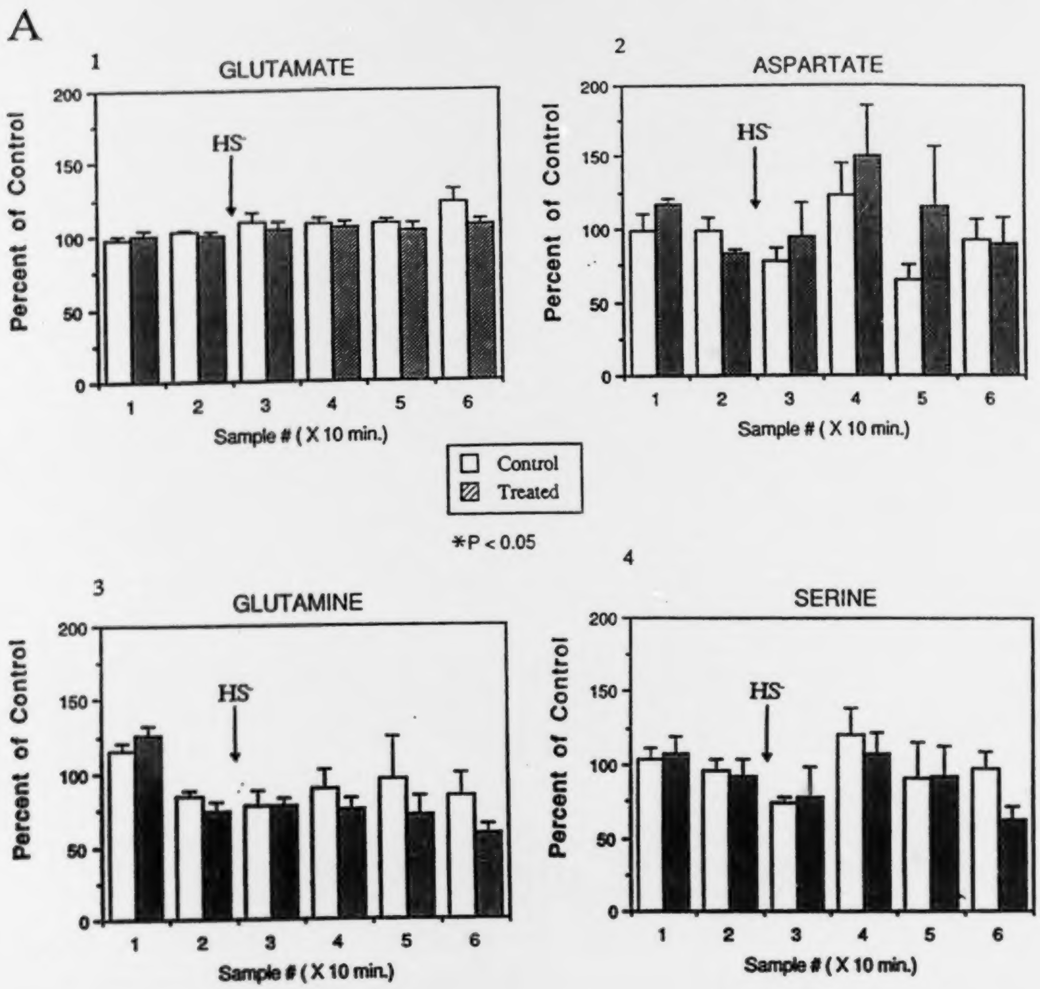
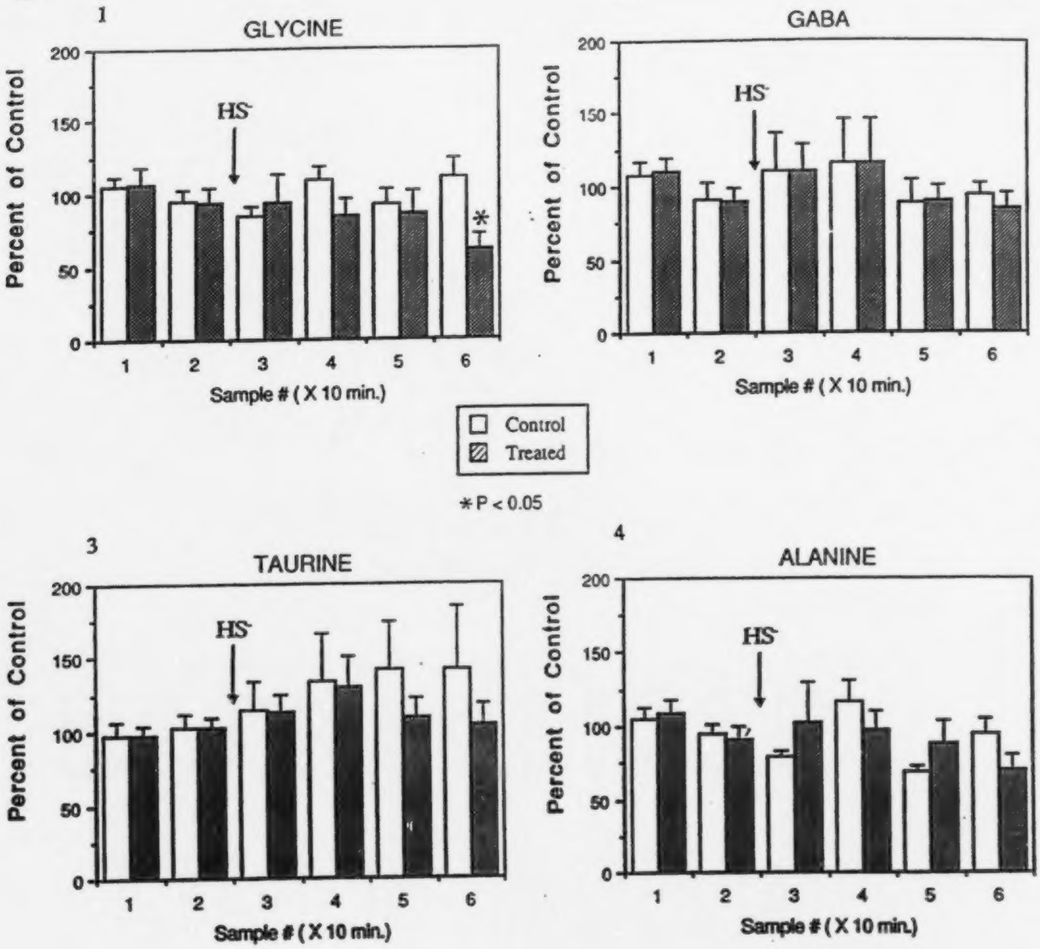


FIGURE 22. Direct, 2 min perfusion (at the rate of 20 ml/min) of the **reticular nucleus** causes a delayed decrease in the amount of glycine in the recovered perfusate. A. 2 min perfusion of a toxicologically relevant concentration of HS^- (3 μg/ml) caused no change in the release pattern of excitatory amino acids in this respiratory related nucleus. Continued next page.

B



B. Only a delayed decrease in the release pattern of glycine alone was detected following the above treatment. The other inhibitory amino acids did not show detectable changes. N=6 in both groups, other details as in Fig. 16.

III. FROG SYMPATHETIC GANGLIA STUDIES (SUCROSE-GAP)

The objective of this study was to examine, on a preliminary basis, the effects of HS^- on neuronal electrophysiological properties and its interaction with conventional neurotransmitter-mediated responses. The frog sympathetic ganglia preparation was a good and convenient tissue of choice to test the hypothesis that sulfide will affect neuronal membrane properties and also modulate transmitter mediated responses, because most of the membrane properties and the transmitter-induced responses of these cells have been characterized (Horn and Dodd, 1981; Rafuse and Smith, 1986; Rafuse et al., 1988). This hypothesis was tested by applying high concentrations of HS^- to sympathetic ganglia and recording the changes in conductance using the sucrose gap recording technique.

a. Effect of Sulfide on Resting Membrane Potential.

In all experiments (n=21), the application of 1.8 mM HS^- produced a membrane depolarization averaging 2.75 ± 0.5 mV ($p< 0.001$; Figs. 23-26). This effect occurred rapidly (within 2 min) and disappeared rapidly following removal of HS^- and was not affected either by reduction in extracellular concentration of K^+, as it was still observed in low K^+ (0.2 mM; Fig. 26), or by a reduction in extracellular Na^+ ion concentrations when NaCl was replaced by sucrose.

b. Effect of Sulfide on Cholinergic Responses.

Acetylcholine is known to produce its effects via activation of two subtypes of

FIGURE 23. Effect of HS^- on depolarizing responses induced by nicotine on frog sympathetic ganglia. A. Depolarizing responses to 30 sec application of 10 μM nicotine; before, during and after 1.8 mM HS^- dissolved in frog Ringer's solution. Application of HS^- to frog sympathetic ganglia induces a depolarizing response. Co-application of nicotine with HS^- did not alter the nicotine response. Nicotine was applied at 15 min intervals. B. Histogram of mean responses to 10 μM nicotine with standard errors. Responses were normalised by comparing all responses to the last control response prior to HS^- application. Significant differences from controls were determined by Duncan's Multiple Range Test at $p< 0.05$.

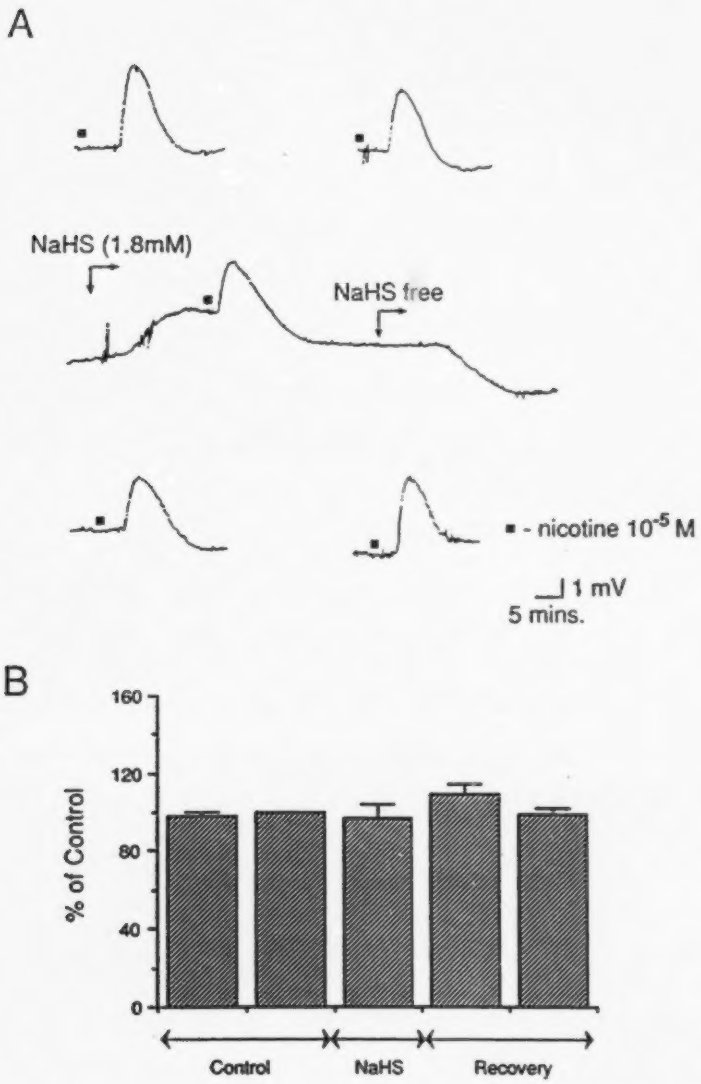
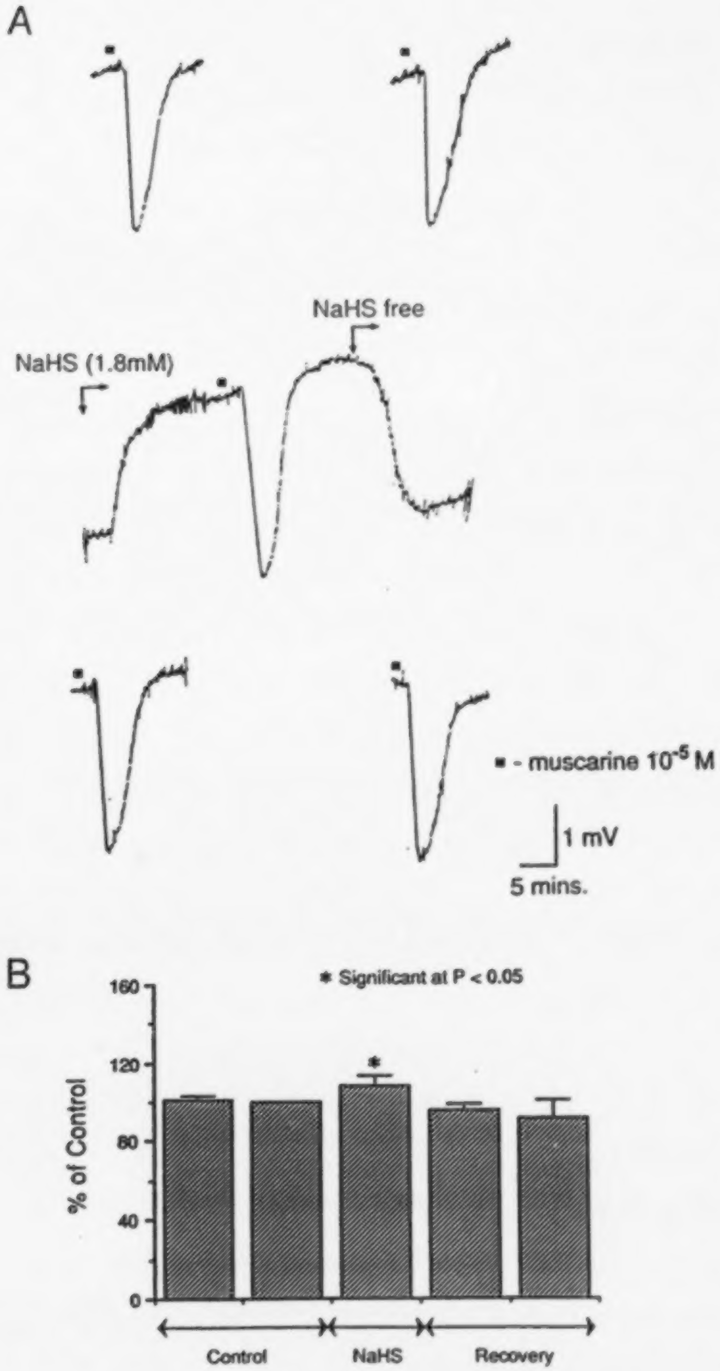

A
NaHS (1.8mM)
NaHS free
■ - nicotine 10^{-5} M
1 mV
5 mins.
B
160
120
80
40
0
% of Control
Control
NaHS
Recovery

FIGURE 24. HS^- potentiates the hyperpolarizing responses induced by muscarine in frog sympathetic ganglia. A. Responses to 30 sec application of 10 μM muscarine; before, during and after exposure to 1.8 mM HS^- dissolved in normal frog Ringer's solution. Drugs were added at 15 min intervals. B. Histogram showing the mean responses to muscarine, other details as in fig. 23.


A
NaHS free
NaHS (1.8mM)
▪ - muscarine 10^-5 M
1 mV
5 mins.
B
* Significant at P < 0.05
160
120
80
40
0
% of Control
*
Control
NaHS
Recovery

receptors, nicotinic and muscarinic receptors. These receptors are selectively activated by nicotine and muscarine respectively. Both of these receptors are present in frog sympathetic ganglia and have been shown to produce different responses in this tissue (Selyanko et al 1988; Rafuse and Smith, 1986).

Nicotine acts on nicotinic cholinergic receptors in this preparation to produce a depolarization which, by its rapid occurrence and fast kinetics, has been regarded as being responsible for fast ganglionic transmission in these cells (Weight, 1983). The fast depolarizing response induced by 10 μM nicotine was not significantly altered by co-application of 1.8 mM HS^- ($p > 0.05$, $n=7$, Fig. 23). Furthermore, in a few experiments, HS^- did not affect orthodromically evoked nicotinic epsps.

Muscarine on the other hand produces a slow, hyperpolarizing response in these cells (Weight and Padjen, 1973; Weight and Smith, 1980, Horn and Dodd, 1981). The responses evoked by application of 10 μM muscarine were reversibly increased (to 108.8 ± 5.2%, $p < 0.05$, $n=14$) following co-application of 1.8 mM HS^- (Fig. 24).

c. Effect of Sulfide on Adrenaline-induced responses.

Adrenaline, instead of noradrenaline appears to be the neurotransmitter in frog sympathetic systems (Falck, Haggendal and Owman, 1963). Application of adrenaline on these neurons produced a hyperpolarization through activation of α_2 adrenoceptors (Brown and Caufield, 1979; Rafuse and Smith, 1986). This hyperpolarization was potentiated in the presence of HS^-. The response of these

FIGURE 25. HS$^-$ potentiates the hyperpolarizing response produced by adrenaline in frog sympathetic ganglia. A. Responses to 30 sec application of 1 μM adrenaline; before, during and after exposure to 1.8 mM HS$^-$ dissolved in normal frog Ringer's solution. Adrenaline was applied at 15 min intervals. B. Histogram showing the mean responses to adrenaline. All other details as in fig. 23.

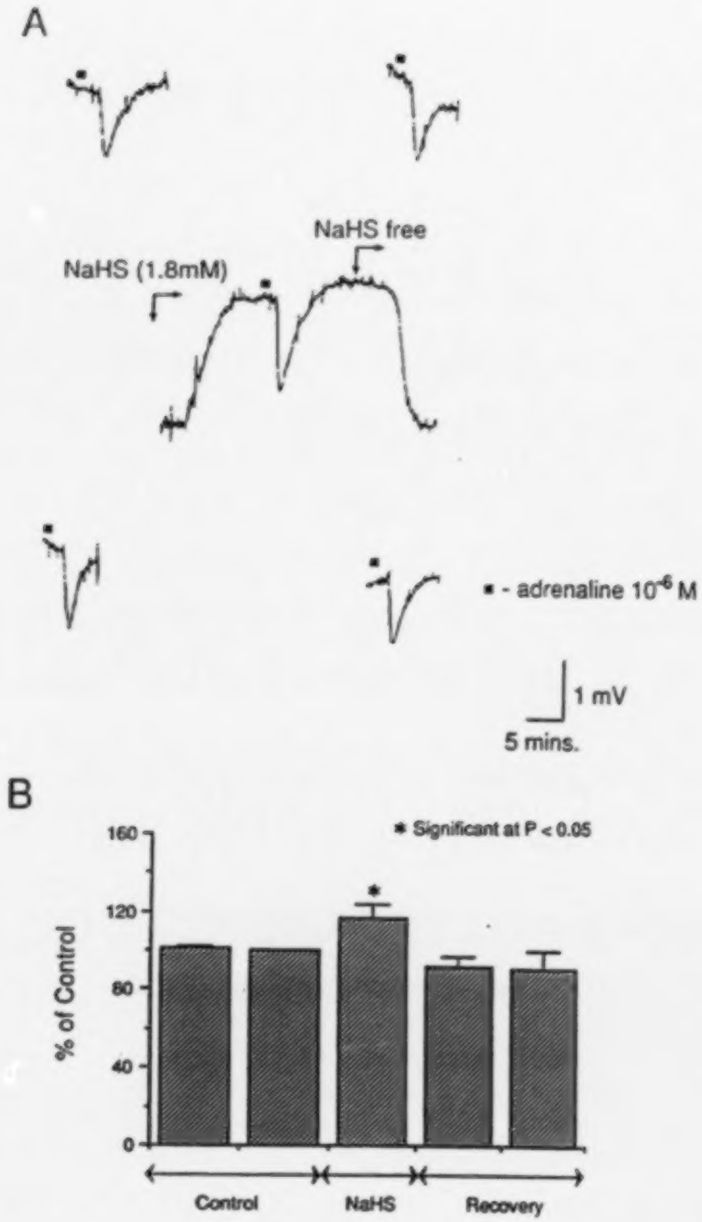
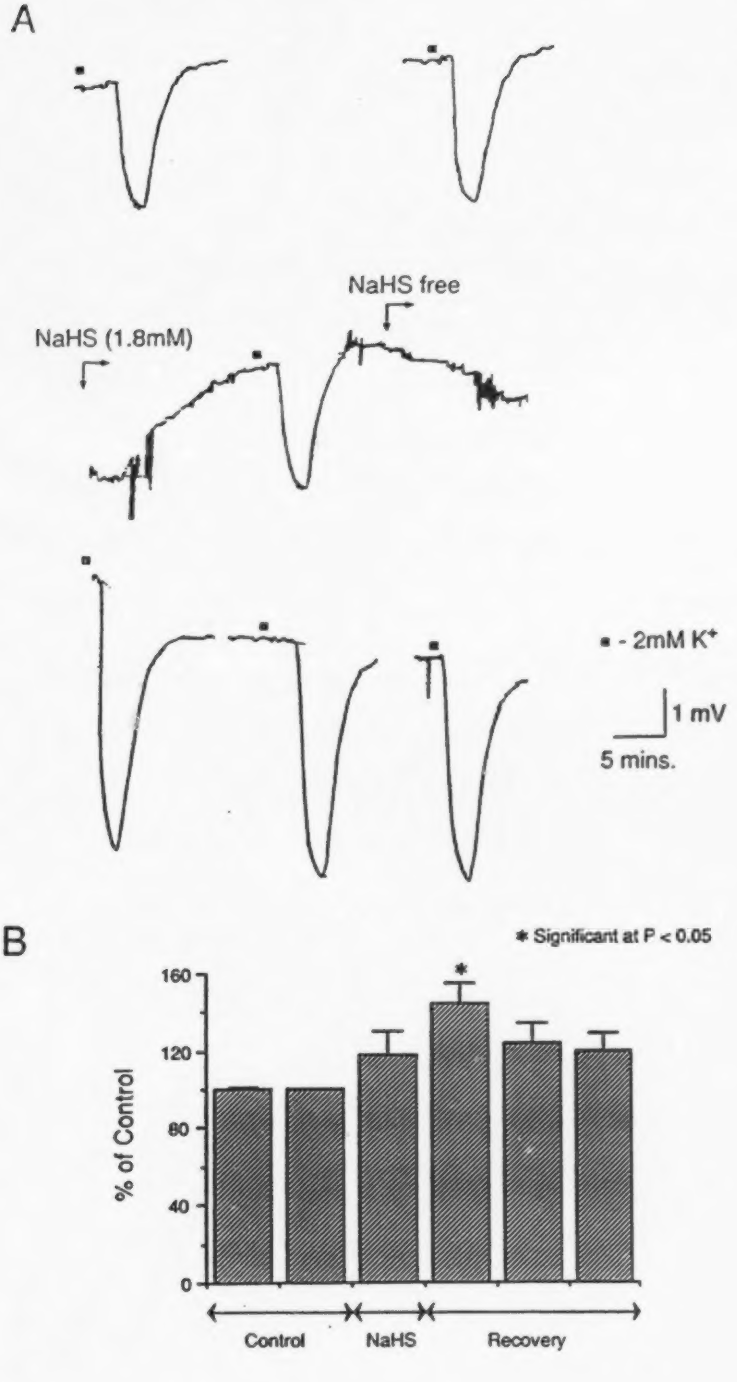

A
NaHS (1.8mM)
NaHS free
■ - adrenaline 10^{-6} M
1 mV
5 mins.
B
* Significant at P < 0.05
160
120
80
40
0
% of Control
*
Control
NaHS
Recovery

FIGURE 26. Effect of HS^- on K^+-induced responses (Na^+/K^+ ATPase pump effect) in frog sympathetic ganglia. Incubation of sympathetic ganglia in low extracellular K^+ (0.2 mM) results in inactivation of the pump, which can then be reactivated by application of normal frog Ringer's solution ($K^+ = 2$ mM) with the production of a hyperpolarization because the pump is electrogenic. A. Responses to 90 sec application of 2 mM K^+ Ringer's solution to sympathetic ganglia incubated in low K^+. Application of HS^- (1.8 mM) still produced a depolarization but did not alter the magnitude of the K^+ activated hyperpolarization. The recovery response following HS^- exposure was potentiated. B. Histogram of the mean responses to 2 mM K^+. All other details as in fig. 23.


A
NaHS free
NaHS (1.8mM)
▪ - 2mM K+
1 mV
5 mins.
B
* Significant at P < 0.05
*
160
120
80
40
0
% of Control
Control
NaHS
Recovery

neurons to 1 μM adrenaline was reversibly increased to 117.1 ± 6.4% of control ($p < 0.05$, $n=7$, Fig. 25) in the presence 1.8 mM HS^-.

d. Effect of Sulfide on Na^+/K^+ ATPase Pump.

As the Na^+/K^+ ATPase pump is important in the generation and maintenance of resting membrane potentials in most excitable tissue, and since HS^- has been demonstrated to inhibit ATPase *in vitro* (Khan, 1989) we were interested in knowing the effect of HS^- on pump-dependent conductances. The removal of K^+ ions from the extracellular solution inactivates the Na^+/K^+ ATPase pump which can then be reactivated by introducing Ringer's solution with normal concentration of K^+ (Smith et al., 1988; Akasu et al., 1975). Since this pump is electrogenic (pumping 3 Na^+ out of the cell for 2 K^+ ions in), its reactivation results in a hyperpolarization of the ganglionic cells. Application of 1.8 mM HS^- in low K^+ still caused a depolarization. However, reactivation of the pump in the presence of HS^- (1.8 mM) did not affect the K^+-activated hyperpolarization ($p > 0.4$, $n=7$; Fig. 26), but upon washout, the first recovery response (15 min post HS^-) was potentiated to 144.4 ± 10% of control ($p < 0.05$, $n=7$; Fig. 26).

IV. *IN VITRO* ELECTROPHYSIOLOGICAL STUDIES IN THE BRAIN STEM.

Paralysis of central respiratory drive has long been thought to underlie HS^- toxicity following exposure to lethal quantities (Evans, 1967; Ammann et al., 1986). However, the mechanism by which sulfide produces this effect is unknown. In previous neurochemical experiments, most changes in response to HS^- application were observed in the brainstem (Kombian et al., 1988; Warenycia et al., 1989). The generation and maintenance of respiratory rhythm is from several nuclei scattered in the brain stem (see Introduction). Detailed electrophysiological characterization of these cells has been difficult, due in part to the diffuse organization of these nuclei (Bystrzycka and Nail, 1985). Furthermore, the size of the cells in most of these nuclei are relatively small in diameter (10 -20 μM) making intracellular recording from them difficult. Most electrophysiological data from these nuclei are therefore, mainly extracellular records of firing patterns in relation to peripheral respiratory muscle activity (Feldman, 1986; Greer et al., 1991). Therefore, to study the detailed mechanism(s) by which sulfide may alter the electrophysiological properties of cells directly, one needs to use readily accessible cells whose electrical properties are well known and with well characterized synaptic events, but which are very similar, in most respects, to those directly involved in the generation and maintenance of respiratory rhythms.

The dorsal raphe nucleus is a midbrain pontine nucleus that contains cells that synthesize, store and release mainly serotonin (5-hydroxytryptamine, 5-HT; Anden et al., 1986; Azmitia and Segal, 1978). It receives synaptic inputs from several other nuclei including other serotonergic nuclei (Aghajanian and Wang, 1977; Yoshimura et al., 1985; Kalen et al., 1986). In addition to having reciprocal connections with regions that are implicated in the control of respiration, this region has also been shown to modulate respiration (see Introduction) and is in close proximity to nuclei involved in respiratory rhythm generation and/or maintenance (also located in the medulla/pons). The dorsal raphe nucleus is a well organised, compact structure and contains cells with excellent physical properties. This makes them amenable to *in vitro* techniques and a potential for performing extensive electrophysiological and pharmacological manipulations (Dingledine, 1984). Finally, the neuronal and synaptic properties of DR neurons have been well characterised both *in vivo* (Baraban and Aghajanian, 1980; Baraban et al., 1978; de Montigny and Aghajanian, 1977; Gallager and Aghajanian, 1976) and *in vitro* (Pan and Williams, 1989; Yoshimura and Higashi, 1985; Williams et al., 1988; Yoshimura et al., 1985; Bobker and Williams, 1990). The complex synaptic responses elicited in these cells by focal electrical stimulation are produced by several neurotransmitters, further providing an unique preparation for examining the interaction of HS^- with different neurotransmitters (see Methods for description of synaptic responses).

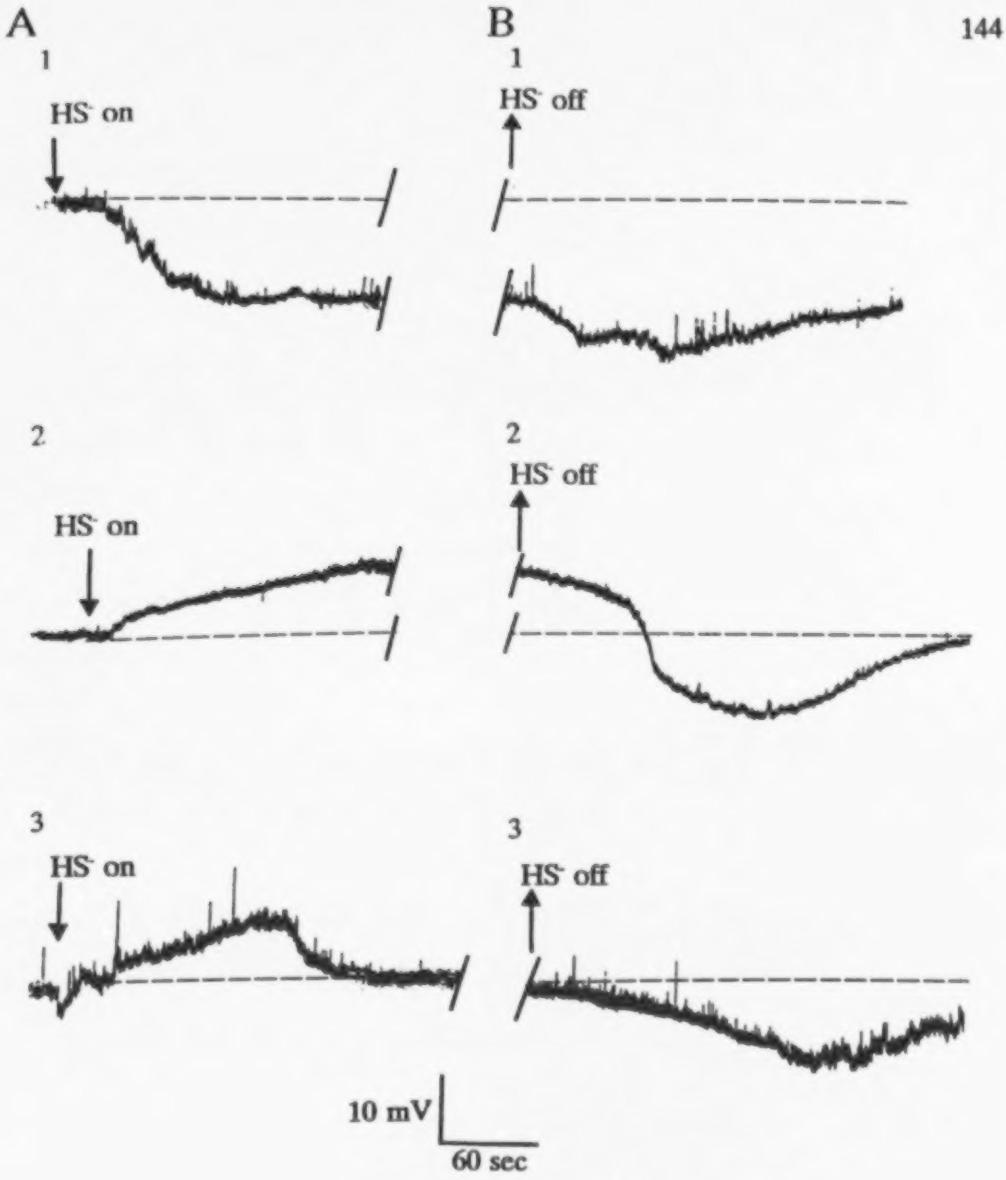
For all the above reasons, the DR neurons were used as model brain stem neurons. The actions of HS^- on these cells were examined to determine whether there were effects consistent with a paralysis of respiratory drive. All cells used for experiments were identified as DR serotonergic neurons using established criteria (Williams et al., 1988, see Methods).

A. EFFECT OF SULFIDE ON POSTSYNAPTIC MEMBRANE PROPERTIES OF DR NEURONS

a. Current Clamp Studies.

HS^- has previously been shown to produce mainly a hyperpolarization in hippocampal CA1 cells (Baldelli, 1990) and a depolarization in frog sympathetic ganglia (Kombian et al., 1988). We therefore wanted to know if HS^- produced similar effects in DR neurons held in current clamp. To test the hypothesis that HS^- would alter the resting membrane conductance of DR neurons, 30-300 μM HS^-, which are toxicologically relevant concentrations (IP injection of the LD_{50} dose of 15 mg/kg produces an equivalent of approximately 75 μM acid-labile S^{-2} in the brain; Warenycia et al., 1989) were applied to impaled DR cells held in current clamp and the changes in membrane potential recorded. Concentrations of HS^- below 30 μM did not produce any measurable change in membrane potential. At 30 μM concentrations of HS^- and greater, changes in membrane potentials were recorded that were concentration-dependent. Four different effects of HS^- on membrane potential were consistently recorded in DR neurons (Fig. 27). (1) A hyperpolarizing response (Fig. 27A1), (2) a depolarizing response (Fig. 27A2) and (3) a biphasic response in which an initial depolarization was superceded by a sustained hyperpolarization (Fig. 27A3). The fourth group of cells consistently did not respond to HS^- even at higher doses (not shown, but see Fig. 29D). In most of these cells with postsynaptic responses to HS^-, a hyperpolarization was observed approximately 1-3 min following the washout of HS^- (Fig. 27B1-3). The hyperpolarizing response peaked in 30-60 sec and returned

FIGURE 27. Sulfide induces four different postsynaptic responses in DR neurons held in current clamp at rmp (-55 to -75 mV). A1. Representative chart record of the group of cells that consistently responded to HS^- (200 µM) with a hyperpolarization. A2. Chart record showing the depolarizing response to HS^- and A3. A third group responded to HS^- with a biphasic response, first a depolarization that was superceded by a sustained hyperpolarization. The last group of DR cells did not have a postsynaptic response to HS^- even at high concentrations (not shown). B1-3. In all DR cells that had a postsynaptic response to HS^-, washout of HS^- was frequently followed, in 1-3 min, by a hyperpolarization which rapidly peaked and returned to baseline.


A
B
1
HS- on
1
HS- off
2
HS- on
2
HS- off
3
HS- on
3
HS- off
10 mV
60 sec

to resting potential. The amplitude of this also depended on HS^- concentration.

These postsynaptic effects of HS^- could either be due to a direct action on postsynaptic membrane; indirectly, through the elicitation of transmitter release from presynaptic terminals which in turn could mediate the observed postsynaptic effects; or via both mechanisms. To test the hypothesis that HS^-'s postsynaptic actions are mediated by the local release of neurotransmitters, we applied antagonists to the receptors known to be present on the DR neuron and examined if HS^- still induced the predetermined response. A "cocktail" containing supramaximal concentrations of glutamate and GABA receptor antagonists; CNQX, 10 μM; APV, 50 μM; picrotoxin, 100 μM was applied. Also included in this cocktail were the α_1 adrenoceptor antagonist, prazosin, 100 nM; and the 5-$HT_{1A\&B}$ receptor antagonist, cyanopindolol, 1 μM. The cocktail eliminated the evoked synaptic response (Fig. 28B2). Application of 200 μM HS^- in the presence of the antagonists resulted in postsynaptic membrane effects smaller than that observed in control (Fig. 28B2); approximately 60% of control in 2 cells; this cell had a biphasic response to sulfide). There was no difference in the washout hyperpolarization between control and in the presence of the "cocktail".

b. Voltage-Clamp Studies.

Because the current clamp technique is not amenable to extensive biophysical manipulations (Kuffler et al., 1984) the voltage clamp technique was applied to examine the detailed mechanism(s) underlying these postsynaptic actions of HS^-. Bath

FIGURE 28. Effect of transmitter antagonists on HS^--induced changes in postsynaptic resting membrane potentials. A. Chart records of the resting membrane potential of a DR neuron impaled with a 2 M KCl-filled microelectrode and held in current clamp. Arrows indicate points where synaptic responses were triggered. Application of 200 μM HS^- induces a biphasic response in this cell, a depolarization followed by a hyperpolarization with a simultaneous attenuation of the synaptic response. Washing out HS^- is followed (1-3 min) later by a washout hyperpolarization. B. 15 min washing in ACSF returns the resting membrane potential to initial levels with substantial recovery of the synaptic potentials. 20 to 30 min application of a "cocktail" containing antagonists to the receptors of the amino acids mediating the depolarizing synaptic potential (6-cyano-7-nitroquinoxalone-2,3-dione, CNQX, 25 μM; DL-2-amino-5-phosphonovaleric acid, APV, 50 μM; and picrotoxin, 100 μM); the $5\text{-HT}_{1A\,\&\,1B}$ receptor antagonist, cyanopindolol, 1 μM, and the α_1 adrenoceptor antagonist, prazosin, 100 nM; completely abolished the synaptic responses. Co-application of HS^- with the "cocktail" of antagonists still produced a biphasic response, although somewhat reduced in magnitude, followed by a washout hyperpolarization after removal of HS^-.

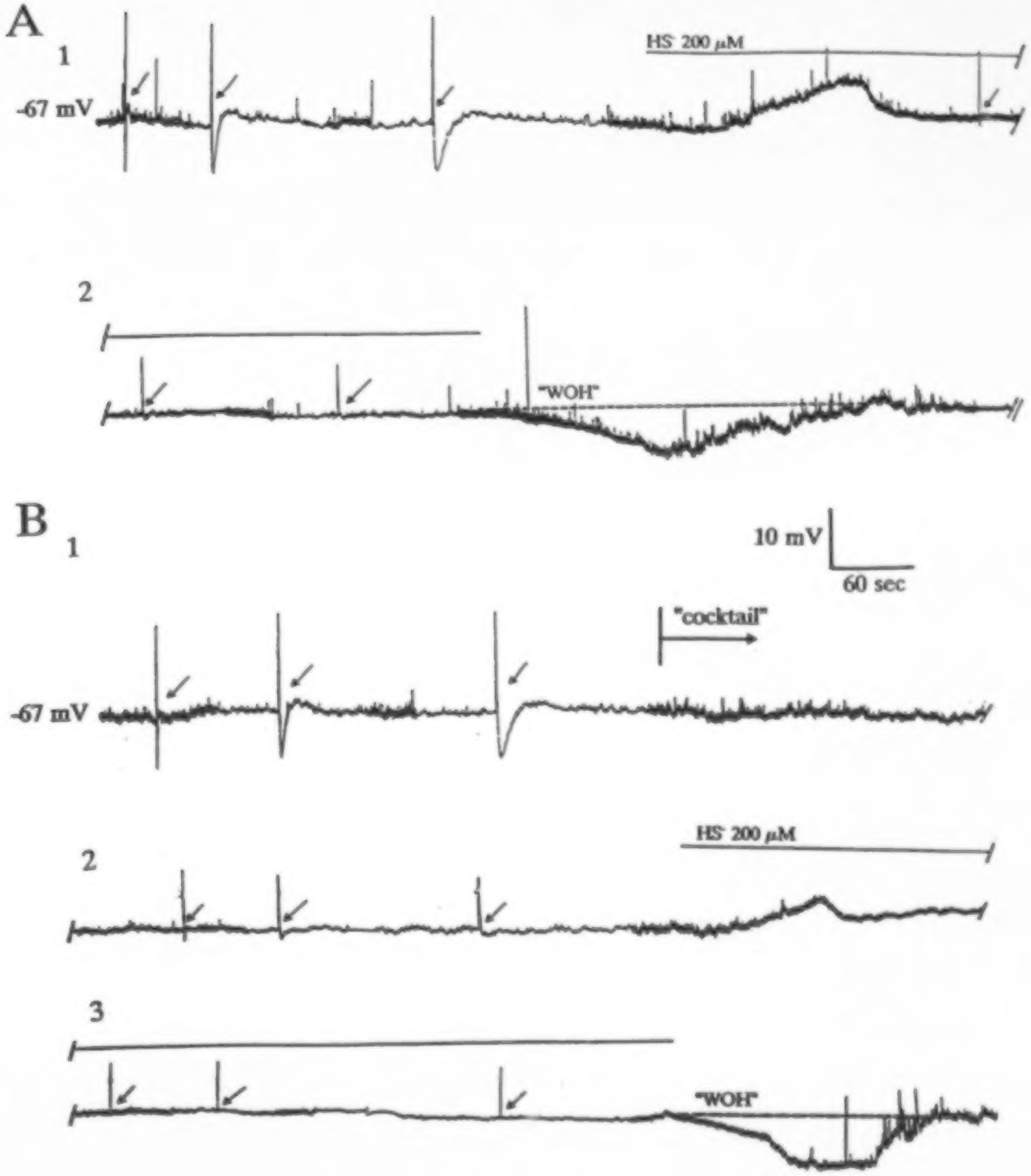
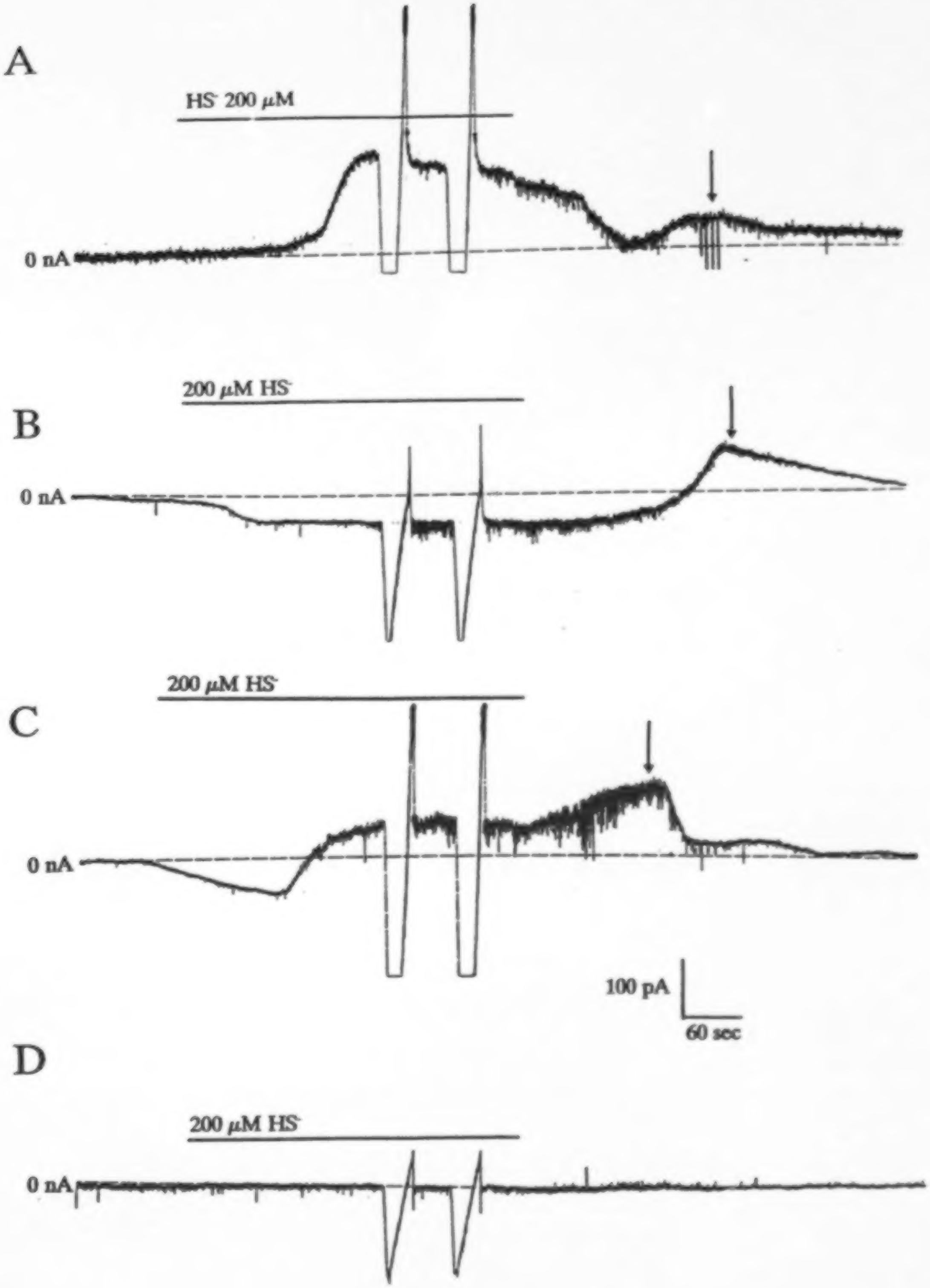

A
1
-67 mV
HS⁻ 200 μM
2
"WOH"
B
1
10 mV
60 sec
"cocktail"
-67 mV
2
HS⁻ 200 μM
3
"WOH"

FIGURE 29. Representative effects of HS^- (200 μM) on DR neurons. A. Application of HS^- to DR neurons voltage clamped near rest (V_h= -60 mV) induces a relatively slow onset (long latency = 90 sec) outward current, which peaks rapidly and remains at peak levels for long periods, in 13.8% of cells studied. Removal of HS^- is followed (1-3 min) by a washout outward current (see arrow). B. In 43.1% of cells studied, HS^--induces a short latency (< 30 sec) inward current which peaks rapidly and is maintained at peak levels for long periods. Removal of HS^- is followed (in 1-3 min) by a robust washout outward current (see arrow). C. 30.3% of all DR cells studied responded to HS^- with an initial short latency (< 30 sec) inward current, lasting approximately 90 sec, which is then superceded by an outward current which peaks in about 120 sec and remains at the peak level for as long as HS^- is present. Removal of HS^- is followed (in 1-3 min) by a washout outward current (see arrow). D. Of all the DR neurons studied, 12.8% had no response to HS^-, even at relatively high concentrations and prolonged periods of application of HS^-. The two large changes in current in all traces were due to the voltage ramps being applied to the cells for analysis of the steady-state current-voltage relationship (see Fig. 32). Current deflections at the peak of the washout current (A) represent voltage steps applied to determine the voltage dependence of this current (see Fig. 45)


A
HS^- 200 μM
0 nA
200 μM HS^-
B
0 nA
200 μM HS^-
C
0 nA
100 pA
60 sec
D
200 μM HS^-
0 nA

application of HS^- to DR neurons held near rest in voltage clamp (V_h= -60 mV) also produced one of four different effects on resting membrane currents.

(1) HS^- induced (in 13.8% of all cells examined) an outward current (hyperpolarization in current clamp) which was slow in onset (latency ≈ 90 sec), peaked in 3-4 min and was accompanied by a 4-fold increase in membrane conductance (from 4.82 ± 0.54 to 21.25 ± 4.90 nS; $p < 0.02$, n=10) determined as the slope conductance between -70 to -55 mV. This conductance reversed at -86.72 ± 3% ($p < 0.001$, n=8; Fig. 29A).

(2) In 43.1% of cells, HS^- induced a relatively fast onset (latency < 30 sec) inward current with very little or no change in conductance. This current peaked in 2-3 min and remained at this peak level for as long as HS^- was present (Fig. 29B).

(3) A third group of DR cells (30.3%) responded to HS^- with a fast onset (< 30 sec) biphasic response; first an inward current (lasting 1-2 min) which was then rapidly superceded by a sustained outward current that peaked in 3-4 min from the time of application (Fig. 29C).

(4) The last group of cells (12.8%) did not respond to HS^- even at concentrations as high as 300 μM and during prolonged periods of HS^- application. This last group are referred to as "nonresponders" (Fig. 29D). The above groups corresponded well with the different responses observed in current clamp.

All the above postsynaptic membrane effects of HS^- (1-3) were concentration-dependent (Fig. 30) with no effect at concentrations below 30 μM, while higher concentrations induced larger membrane currents as shown by the concentration-

FIGURE 30. HS$^-$ effects on DR neurons are concentration-dependent. A. Chart records of the effect of two concentrations of HS$^-$ (200 and 300 μM) on a DR neuron that responds to HS$^-$ with an outward current. The cell was voltage clamped near rest (-60 mV). B. Chart records of a voltage clamped DR cell which responds to HS$^-$ with an inward current (V_h= -60 mV). 200 and 300 μM HS$^-$ produced different magnitudes of inward current. Time and voltage scales are the same in both A and B.

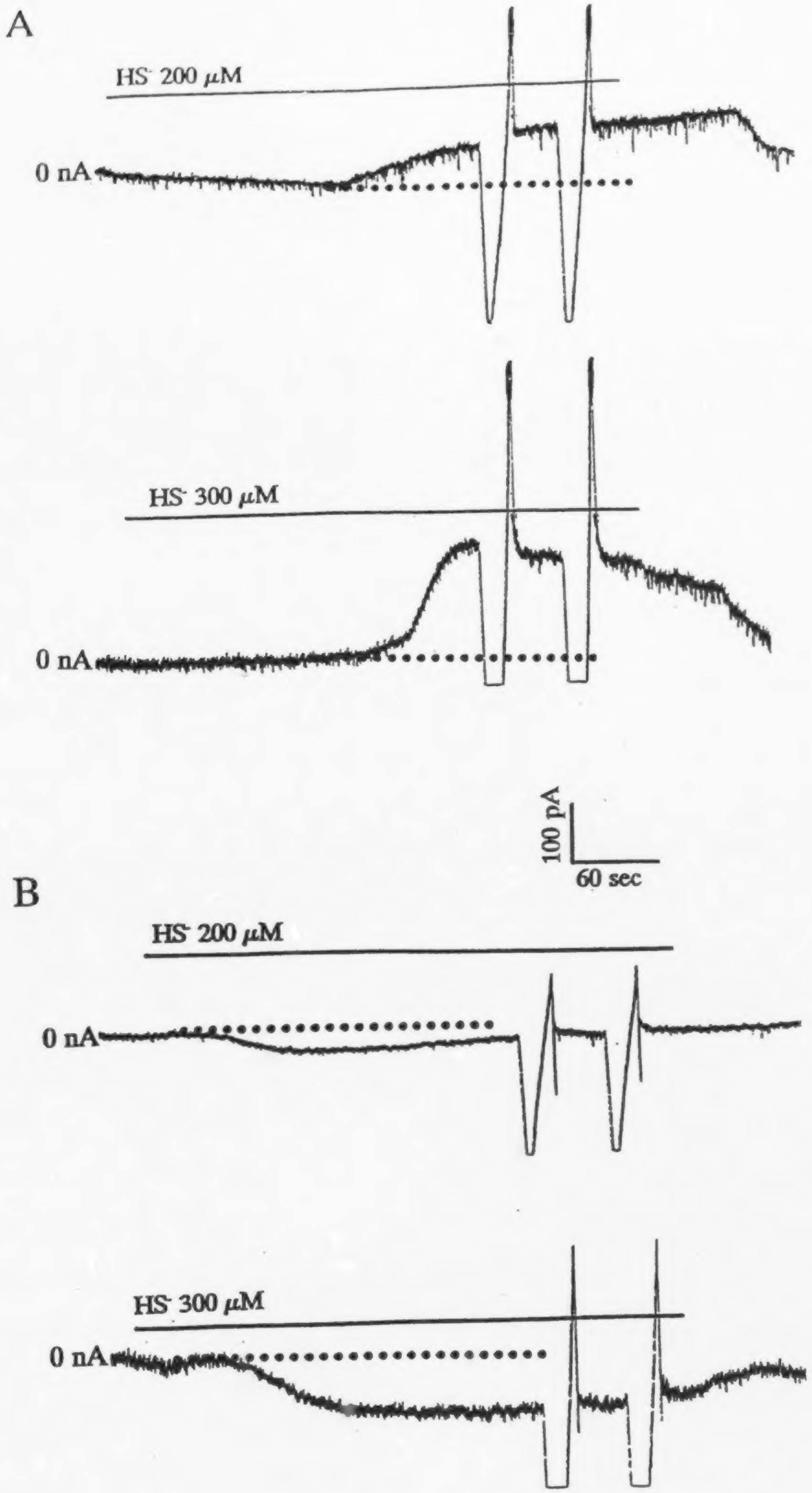
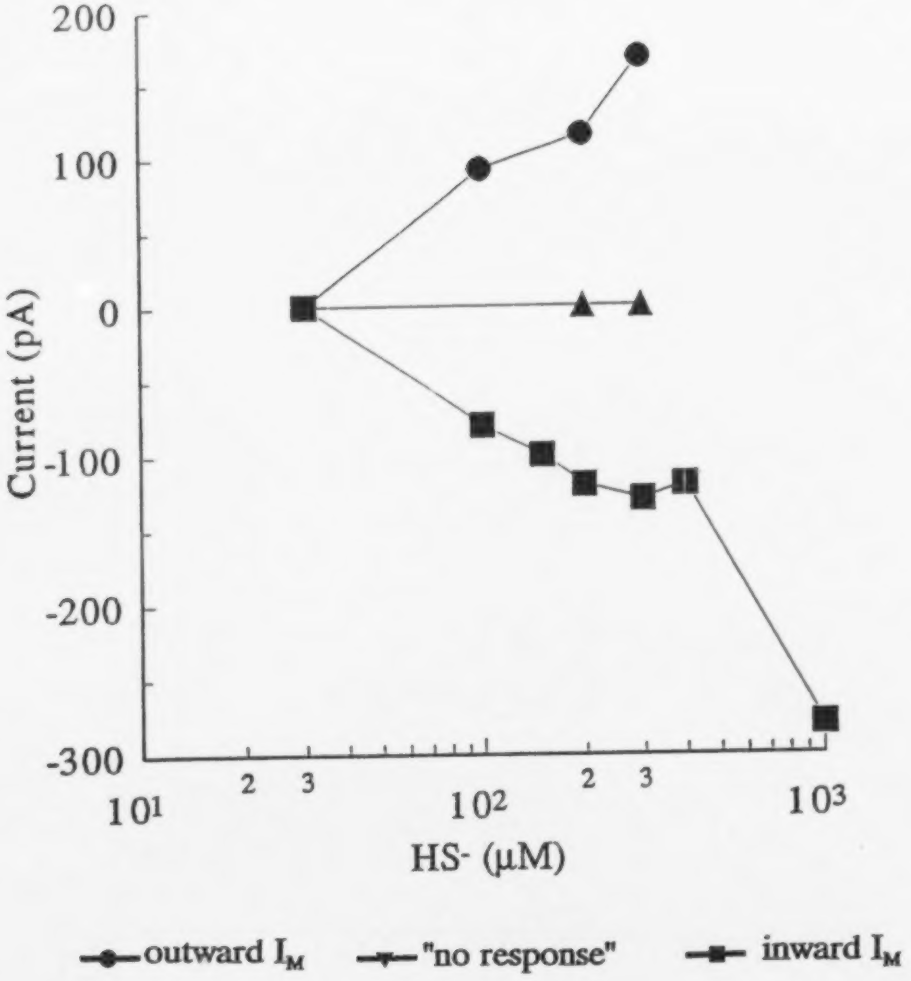

A
HS- 200 μM
0 nA
HS- 300 μM
0 nA
100 pA
60 sec
B
HS- 200 μM
0 nA
HS- 300 μM
0 nA

FIGURE 31. Concentration-response curves showing the concentration-dependent actions of HS^- on postsynaptic membrane currents in 3 different types of DR neurons voltage clamped near rest (V_h= -60 mV). The clamp amplifier passed increasingly positive current into the cell in the presence of HS^- to keep the V_h constant for cells with an outward current response to HS^-. Thus, the curve for cells with outward current response to HS^- increased in the positive direction with increasing concentrations of HS^- (●). In cells that responded to HS^- with an inward current, the clamp amplifier passed increasingly negative current to keep the membrane potential at V_h. Thus, the curve for these cells increased in the negative direction as more and more negative current had to be passed at higher HS^- concentrations to hold the cell at the command voltage (■). For the cells with no postsynaptic response to HS^-, no compensatory current was needed to keep the V_h constant. As such, the curve for these cells is a flat line (▼).


200
100
0
-100
-200
-300
Current (pA)
10^1
2
3
10^2
2
3
10^3
HS- (μM)
outward I_M
"no response"
inward I_M

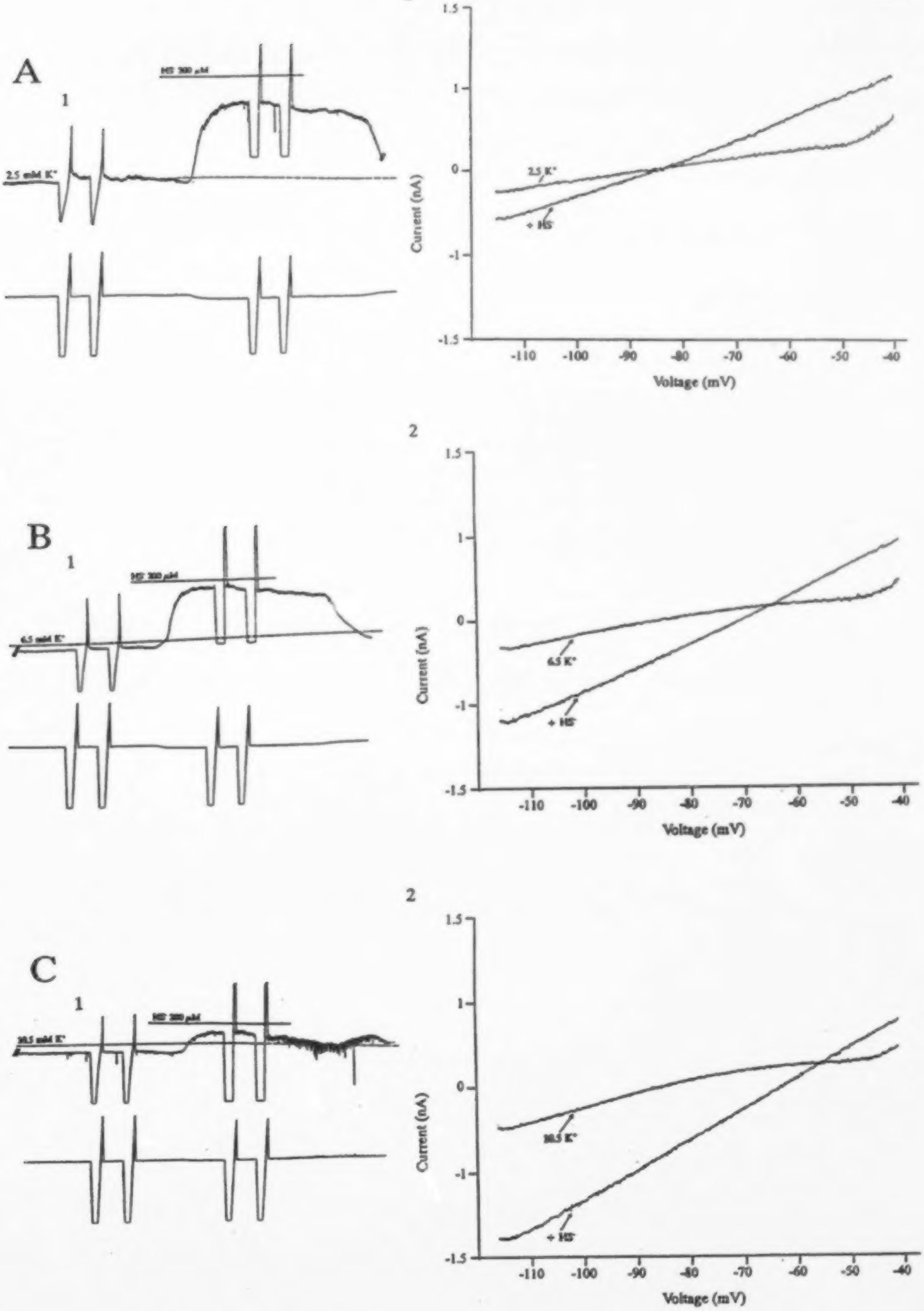
response curve in Fig. 31. Only responses obtained from cells responding to HS^- with outward and inward currents are shown in figure 30.

In most cells that demonstrated any of the first three postsynaptic responses to sulfide above, an outward current was observed approximately 1-3 min following the washout of HS^-, that peaked in about 30-60 sec and rapidly returned to baseline (Fig. 29). This corresponded well with the washout hyperpolarization observed in the earlier current clamp studies.

c. Studies on the Mechanism(s) of the Postsynaptic Actions of Sulfide

To determine the mechanism(s) by which HS^- produced the above membrane currents, voltage-clamp protocols and extracellular solutions appropriate to the analysis of steady-state ionic currents were employed. Steady-state current-voltage relationships (I-V curves) were obtained in the absence and presence of HS^-. In most cells, application of a slow (> 20 sec) voltage ramp from -115 to -40 mV yielded a steady-state I-V curve which exhibited an inward rectification in the more negative voltage range (-90 to -115 mV) and an outward rectification at more positive voltages (-50 to -40 mV). The degree of rectification differed from cell to cell as described by Williams et al. (1988). Following control responses, 200 μM HS^- (this dose was chosen because its effects were pronounced, yet rapidly reversible) was applied for 4-8 min. Data acquisition began once the postsynaptic effect had peaked (about 4-5 min), usually starting with the ramp reponses, then followed by the synaptic responses. In some experiments, synaptic responses were not measured in the presence of HS^-,

FIGURE 32. Effect of changing extracellular K^+ concentration ($[K^+]_O$) on HS^--induced outward current and the steady-state current-voltage (I-V) relationship obtained in a DR neuron, voltage clamped close to rest (V_h= -60 mV), by the application of a slow, staircase depolarizing voltage ramp from -115 to -40 mV. A.1 Upper panel. Chart record of the outward current (hyperpolarization in current clamp) induced by HS^- (200 μM) in a DR neuron. At the peak of the current is the steady-state current (at a slow chart speed of 0.25 mm/sec) produced by the voltage ramp protocol (slowly ramping the cell from V_h to -115 mV in 2 sec, holding it there for a further 2 sec and then ramping it to -40 mV over 20 sec and then back to V_h; see Methods) as shown in the lower panel. A.2 The steady-state I-V relationship obtained from A.1 in normal ACSF (2 mM K^+) and in the presence of HS^- (200 μM). The point of intersection of these two I-V relationships is -87 mV and represents the reversal potential (E_{rev}) of the HS^--mediated conductance increase. The HS^- induced conductance increase was nearly linear (non rectifying) in the entire voltage range studied. B.1 Chart record as in A.1. A change in $[K^+]_O$ to 6.5 mM produced a slight inward current (depolarization) in the cell as shown by the shift in the baseline of the current trace. Application of HS^- (200 μM) produced a much reduced outward current. B.2 The I-V relationship in 6.5 mM K^+ alone and in the presence of HS^- are shown superimposed (E_{rev} = -68 mV). C.1 Chart record as in A.1. Further increase in $[K^+]_O$ to 10.5 mM induces a larger inward current in these cells. Co-application of HS^- (200 μM) produces a much reduced outward current which reversed direction to become an outward current prior to the 4 min HS^- application time (E_{rev} = -59 mV).


A
1
HS⁻ 200 µM
2.5 mM K⁺
2
Current (nA)
Voltage (mV)
2.5 K⁺
+ HS⁻
1.5
1
0
-1
-1.5
-110
-100
-90
-80
-70
-60
-50
-40
B
1
HS⁻ 200 µM
6.5 mM K⁺
2
6.5 K⁺
+ HS⁻
C
1
HS⁻ 200 µM
10.5 mM K⁺
2
10.5 K⁺
+ HS⁻

however, control synaptic responses were always measured for the purpose of identifying the neurons as serotonergic cells (see Methods). HS^- washout was commenced immediately after the last synaptic or ramp response was taken. A recovery period of at least 10-15 min was allowed between each HS^- application; this was sufficient for complete recovery from HS^- effects. When other drugs were used in conjunction with HS^-, they were usually applied until their effect peaked (but see strophanthidin and DTT experiments). HS^- was then dissolved in the same drug solution and applied for the predetermined period (4-8 min).

i. Experiments to Determine the Mechanism(s) of the HS⁻-induced Outward current.

Experiments were performed to determine the mechanism and ionic nature of the outward current induced by HS^-. In neurons, outward currents are often due either to an efflux of K^+ ions from the cell or an influx of Cl^- ions from the extracellular medium into the cell. In this study, most neurons were impaled with glass micro-electrodes filled with 2 M KCl. Under these conditions, the Cl^- ions freely diffuse into the cell resulting in a shift of the Cl^- equilibrium (reversal) potential to more positive voltages (Pan and Williams, 1989; Newberry and Nicoll, 1984 & 1985). Under these conditions, application of HS^- should result in efflux of Cl^- (inward current) if HS^- were activating a Cl^- conductance. However, in these cells, 200 μM sulfide induced an outward current of about 200-500 pA suggesting the activation of a K^+ conductance (Fig. 32A). To test the hypothesis that the outward current induced by HS^- was due to the activation of K^+ channels, slow voltage ramp commands (> 20 sec duration) were applied in the absence and presence of 200 μM HS^- (see Methods;

Fig. 32A1). Comparison of the steady-state I-V relationship obtained in control with that obtained in the presence of HS^- indicates that the outward current was accompanied by an increase in conductance, as the slope of the latter curve (a measure of conductance) was increased (Fig. 32A2). Superimposition of these two curves showed them to intersect at -86.7 ± 3.0 mV ($p < 0.001$, $n=8$; Fig. 32A2) indicating that the current reversed or was at equilibrium at this voltage. This point is referred to as the equilibrium or reversal potential of the HS^--mediated conductance increase. These results suggested that the sulfide-induced outward current was not due to a Cl^- conductance (the reversal potential of which should be more positive than -86 mV), but is more likely due, at least in part, to a K^+ current with a reversal potential around -90 to -105 mV in most cells.

Nernst (1888) derived equations to show that the equilibrium potential of a current depended mainly on the ratio between the extracellular and intracellular concentrations of the ion carrying the current and changed in a predictable manner with every change in this ratio. To further characterize the above current and to obtain more evidence that this current was carried by K^+ ions, the extracellular concentration of K^+ was varied and the HS^--induced current was examined under these conditions (Fig. 32B & C). According to the Nernst equation, if the current is carried by K^+ ions, then the reversal potential (E_{rev}) should shift in the positive direction by a predictable amount. A ten-fold increase in extracellular K^+ ion concentration should produce a predictable 61 mV change in the reversal potential for K^+ at the temperature at which these experiments were done (34°C; Hille, 1991).

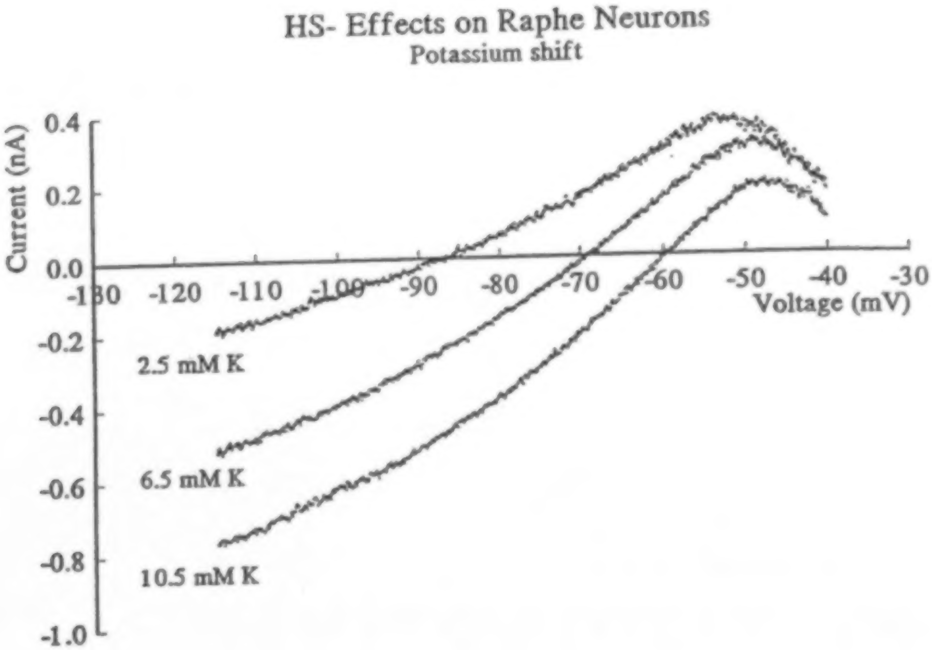
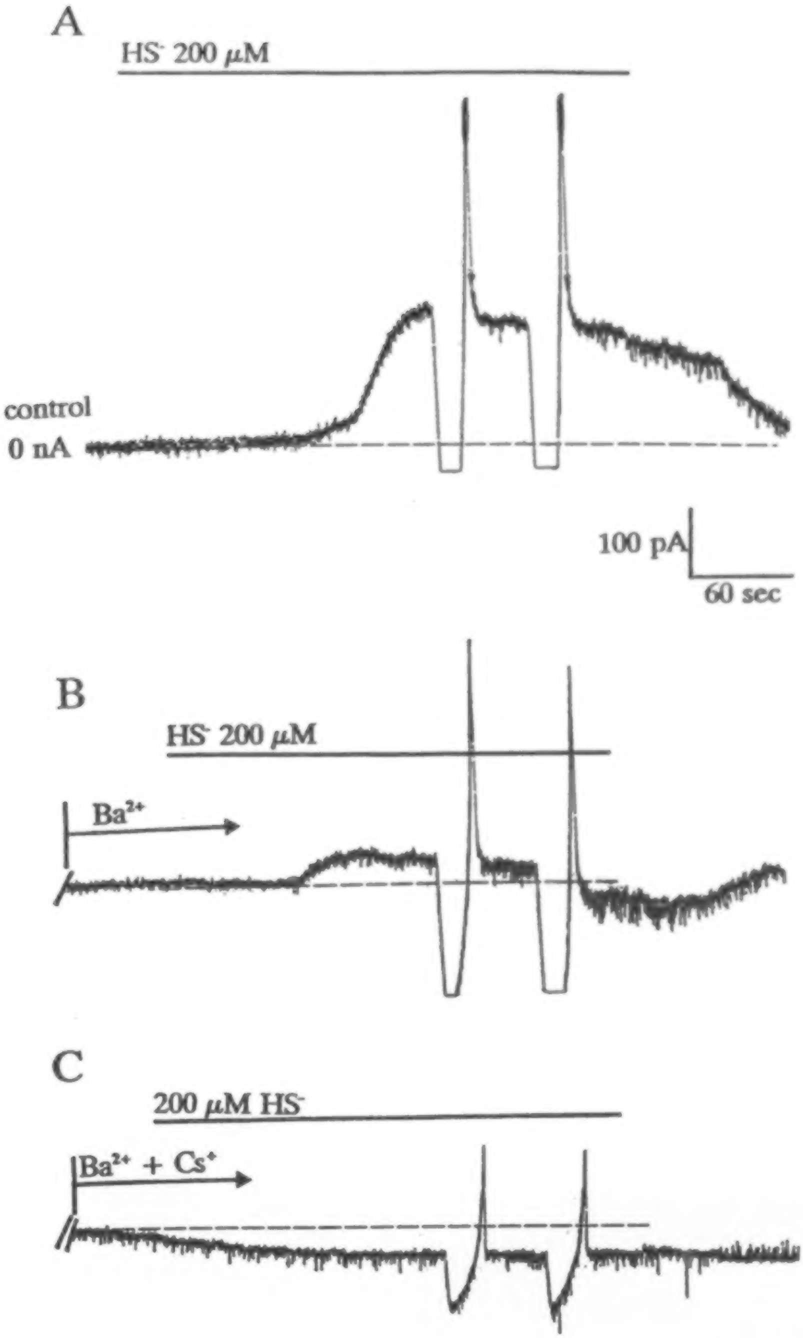


FIGURE 33. Digital subtraction of the I-V curves in figure 32 showing the HS^- induced outward current under different extracellular potassium concentrations. Each curve intersects the zero current line at the reversal or equilibrium potential of the ion carrying the HS^--mediated conductance increase. HS^- in addition, either inhibits an outwardly rectifying conductance or activates an inward current in these cells in the voltage range -50 to -40 mV.

Application of 6.5 mM K^+ (normal K^+ being 2.5 mM) produced a small inward current (depolarization) consistent with a decrease in the electrochemical gradient of K^+ between the intra- and extracellular milieu (Fig. 32B1; also see Williams et al., 1988). Application of 200 μM HS^- in the presence of 6.5 mM extracellular K^+ resulted in a decreased HS^--induced outward current at rest and a shift in the reversal potential to a more positive voltage (Fig. 32B1). In 10.5 mM extracellular K^+, the outward current peaked relatively quickly and reversed direction into an inward current before the 4 min HS^- application time (Fig. 32C1). The voltage ramp responses (I-V curves) taken at the peak of each HS^- application showed a shift in the point of intersection (E_K) in the positive direction with each increase in extracellular K^+ ion concentration (Fig. 32B2 & C2). Digital subtraction of the I-V relationships in Fig. 32 shows the currents induced by HS^- over the entire voltage range tested. Each of these curves intersect the abscissa (zero current) at the equilibrium or reversal potential, showing more clearly the reversal potentials of the current induced by HS^- (Fig. 33).

The theoretical values of these shifts were calculated from the Nernst equation: $E_K = RT/ZF\ \mathit{ln}\ [K^+]_{out}/[K^+]_{in}$. This equation can be rewritten for a monovalent ion and at 34°C in a simplified form: $E_K = 61 \log_{10} [K^+]_{out}/[K^+]_{in}$. For these cells and assuming an intracellular K^+ concentration of 135 mM (Williams et al., 1988), the predicted E_Ks are, -105.7 mV at 2.5 mM; -80.4 mV at 6.5 mM; and -67.7 mV at 10.5 mM (Williams et al., 1988). In these experiments, the measured E_{rev}'s for the HS^--induced outward current were -86.7 ± 3 mV (n=8), -70.9 ± 2.9 mV

FIGURE 34. The HS^--induced outward current is only partially blocked by Ba^{2+} but is completely blocked by a combination of Ba^{2+} and Cs^+. A. Chart record of the resting current of a DR neuron clamped near rest (V_h= -60 mV) and the effect of HS^- (200 μM) on the resting conductance. This cell responded to HS^- with an outward current only. B. 10 min bath application of Ba^{2+} (100 μM) only partially blocks the HS^--induced outward current. C. Addition of 2 mM Cs^+ to the 100 μM Ba^{2+}, not only completely eliminates the HS^--induced outward current, but also ummasks an underlying inward current (see characteristics of this current in Fig. 40).

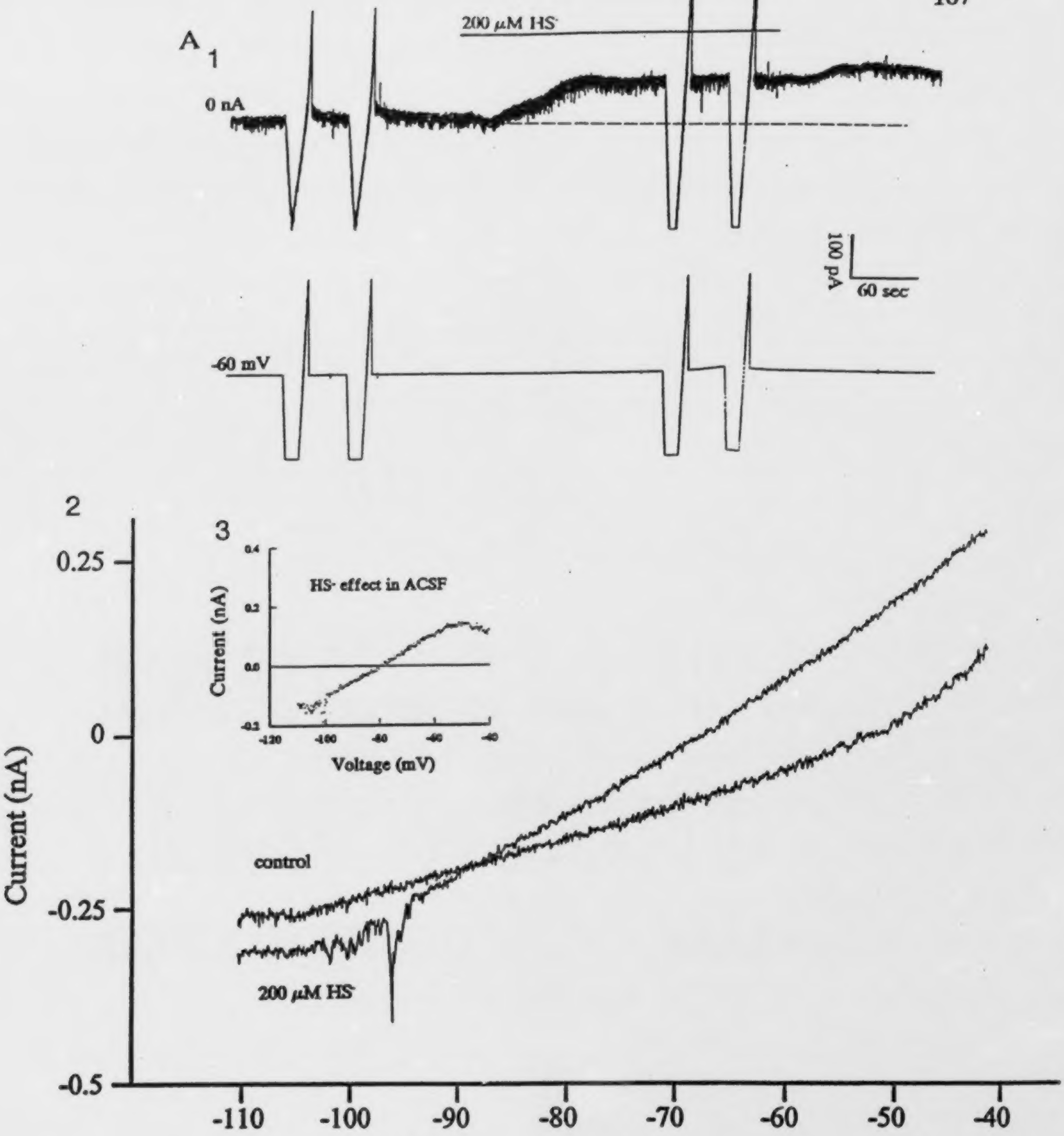

A
HS- 200 μM
control
0 nA
100 pA
60 sec
B
HS- 200 μM
Ba2+
C
200 μM HS-
Ba2+ + Cs+

(n=8), and -64.0 ± 0.9 mV (n=3) at 2.5, 6.5 and 10.5 mM extracellular K^+ concentration respectively (Figs. 32 & 33). These potentials are more positive than the Nernstian predicted values, with the deviation more pronounced at lower extracellular K^+ concentration. A linear regression of the extracellular K^+ ion concentration on the E_K of the sulfide-induced outward current yielded a slope of about 37.

To further determine whether the sulfide-induced outward current was due to activation of a K^+ conductance, selective K^+ channel blockers were applied. Several different K^+ channels have been characterised in various tissues including neurons (see reviews by Rudy, 1988; Armstrong, 1975; Adams et al., 1980; Adrian, 1969) that are blocked by both organic and inorganic compounds or ions, applied extracellularly or intracellularly. As most voltage-dependent K^+ channels, especially the inwardly rectifying ones, have been shown to be blocked by extracellular cesium (Cs^+) and/or barium (Ba^{2+}) ions (Rudy, 1988; Armstrong, 1975; Yellen, 1984; Hagiwara et al., 1976; Hille, 1991; Williams et al., 1988), we tested the hypothesis that the HS^--induced K^+ current was caused by the activation of voltage-sensitive K^+ channels by examining HS^- actions in the presence of Ba^{2+} and Cs^+. Bath application of 100 μM Ba^{2+} produced a slight membrane depolarization or inward current. This concentration of Ba^{2+} (100 μM) only partially blocked the HS^--induced outward current (Fig. 34B). A combination of Ba^{2+} and Cs^+ (2 mM) however, completely blocked this response and in addition, the combination unmasked an underlying inward current (Fig. 34C).

As the actions of Ba^{2+} and Cs^{+} are relatively nonselective (Rudy, 1988; Kolb, 1990; Hille, 1991), affecting most voltage sensitive K^{+} channels, we wanted to know if the action of HS^{-} was specific on only certain K^{+} channels but not others. Of all the K^{+} currents sensitive to these cationic blockers, the Ca^{2+}-activated K^{+} current is most easy to isolate and study as it is activated by intracellular Ca^{2+} which acts as a specific ligand for the channels (see review by Kolb, 1990; Latorre, 1982; Moczydlowski and Latorre, 1983). To test the hypothesis that the HS^{-}-induced K^{+} current was due to activation of a calcium-dependent K^{+} channel, the extracellular Ca^{2+} concentration was markedly reduced from the normal level of 2.4 mM to 0.5 mM. Because reduced Ca^{2+} solutions tend to destabilize the plasma membrane, Ca^{2+} was replaced with 8.8 mM magnesium (Mg^{2+}) bringing its final level to 10 mM. This modified ACSF completely abolished the synaptic currents recorded in these cells indicating a fairly complete blockade of voltage-sensitive Ca^{2+} channels (Fig. 35B3) Subsequent application of 200 μM HS^{-} in the presence of the low Ca^{2+}, high Mg^{2+} ACSF failed to induce the outward current recorded in control (Fig. 35A & B; n=3). Digital subtraction of the I-V curves in control and in the modified ACSF revealed that HS^{-} induced no current over the entire voltage range examined. This blockade of HS^{-} effects was reversible, as both the synaptic responses (Fig. 35B3) and the HS^{-} induced outward current returned after washing with ACSF containing normal Ca^{2+} and Mg^{2+} concentration (not shown). Despite the blockade of the outward current with low Ca^{2+}, Mg^{2+} ACSF, an outward current was observed 1-3 minutes subsequent to the washout of HS^{-}, indicating that other actions of HS^{-} were not affected by the

FIGURE 35. The HS^--induced outward current is also abolished in the absence of extracellular calcium (extracellular Ca^{2+} was reduced to 0.5 mM from 2.4 mM and replaced with Mg^{2+} by increasing extracellular Mg^{2+} to 10 mM from 1.2 mM to produce the "LoCa^{2+} HiMg^{2+}" cocktail). A1. Upper panel. Chart record of resting current and the outward current induced by HS^- (200 μM). Two slow voltage ramps (lower panel) were applied in control and at the peak of the HS^--induced outward current to obtain the steady-state I-V relationships. A2. Superimposed are the steady-state I-V curves from A1 showing the E_{rev} of the HS^--induced conductance increase. Inset A3. Digital subtraction of the curves in A2 showing the HS^--induced current. B1. Substitution of the normal extracellular Ca^{2+} ion concentration (2.4 mM) with a modified ACSF containing "LoCa^{2+} HiMg^{2+}" resulted in the abolition of the HS^--induced outward current. The synaptic current evoked by focal electrical stimulation was monitored in order to determine maximal effect of Ca^{2+} removal (inset B3). Co-application of HS^- produced only a small transient outward current that returned to rest . Subsequent reperfusion with normal ACSF resulted in recovery of synaptic response (inset B3) and recovery of HS^- effect (not shown). B2. The I-V curves from B1 superimposed. Inset B4. Digital subtraction of curves in B2 showing the HS^--induced current in "LoCa^{2+} HiMg^{2+}".


A 1
200 μM HS-
0 nA
100 pA
60 sec
-60 mV
2
0.25
0
-0.25
-0.5
Current (nA)
-110
-100
-90
-80
-70
-60
-50
-40
control
200 μM HS-
3
HS- effect in ACSF
Current (nA)
0.4
0.2
0.0
-0.2
-120
-100
-80
-60
-40
Voltage (mV)

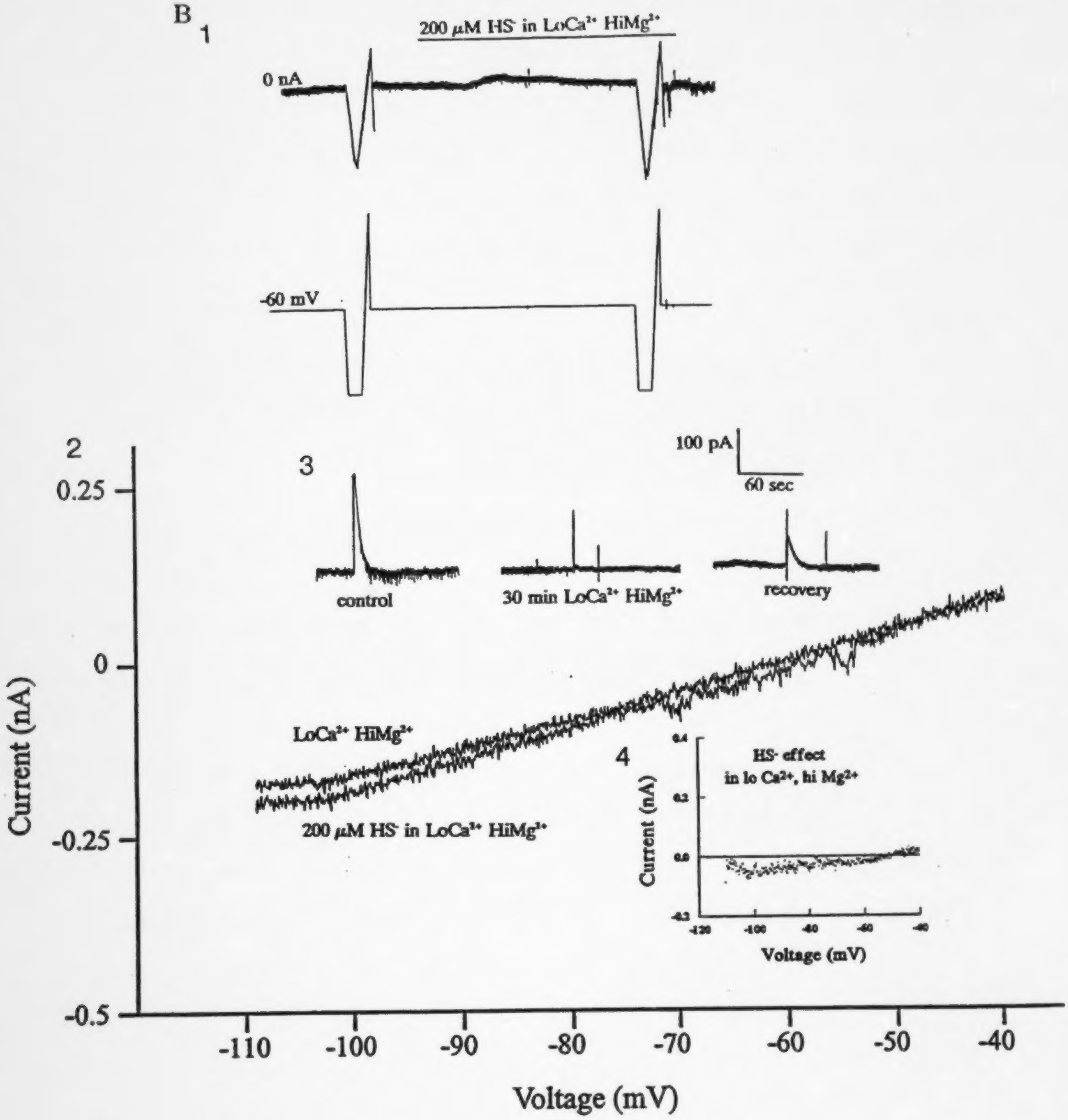
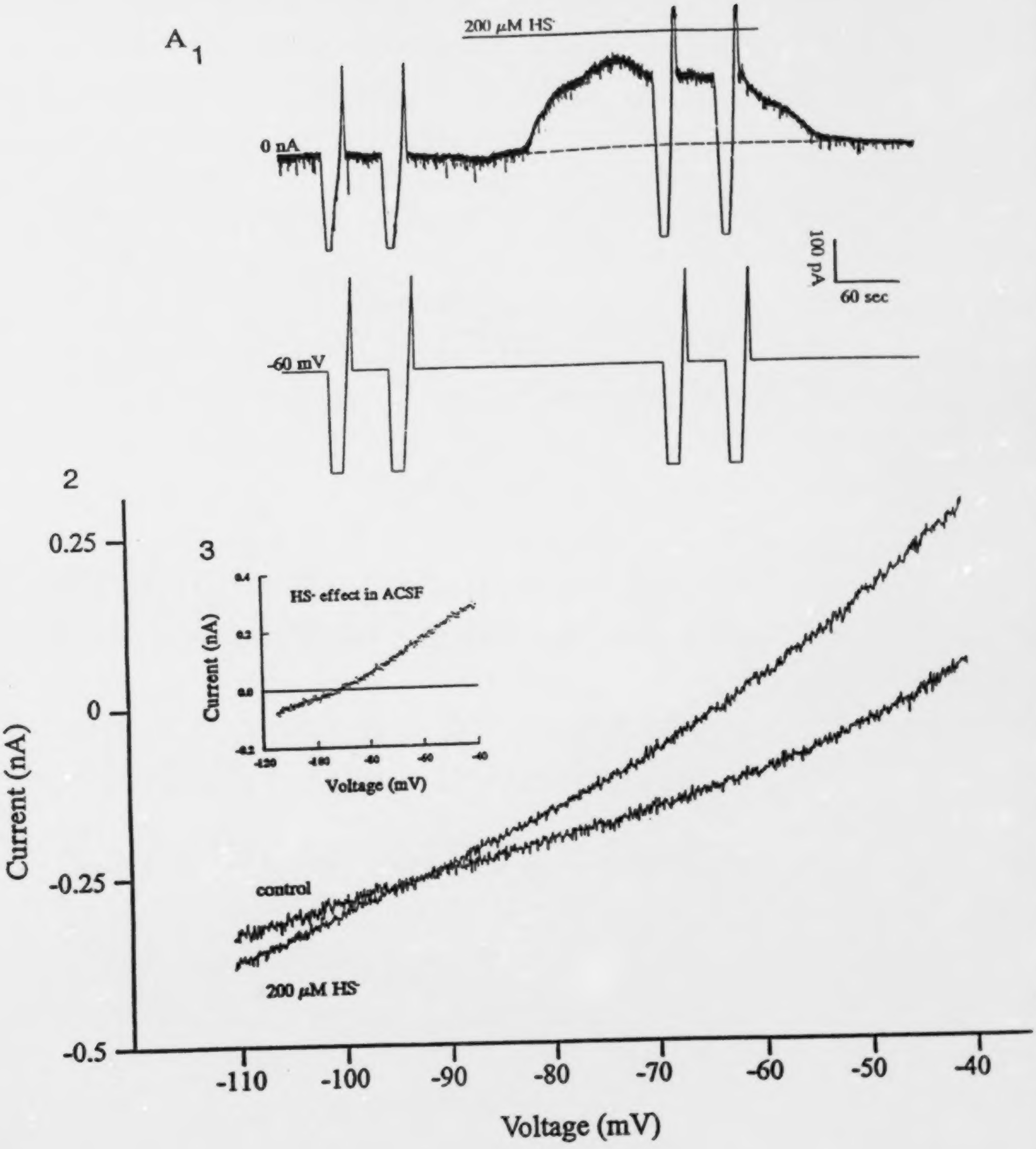

B
1
200 μM HS⁻ in LoCa²⁺ HiMg²⁺
0 nA
-60 mV
2
3
100 pA
60 sec
control
30 min LoCa²⁺ HiMg²⁺
recovery
0.25
0
-0.25
-0.5
Current (nA)
LoCa²⁺ HiMg²⁺
200 μM HS⁻ in LoCa²⁺ HiMg²⁺
-110
-100
-90
-80
-70
-60
-50
-40
Voltage (mV)
4
HS⁻ effect
in lo Ca²⁺, hi Mg²⁺
0.4
0.2
0.0
-0.2
Current (nA)
-120
-100
-80
-60
-40
Voltage (mV)

FIGURE 36. The HS^--induced outward current is abolished by extracellular cadmium (Cd^{2+}; 200 μM), a calcium channel blocker. A1. Upper panel. Chart record of resting current of a DR neuron clamped near rest (V_h= -60 mV) in the whole-cell recording mode, and the effect of HS^- (200 μM) on the resting conductance. Two steady-state I-V relationships were taken in control and in the presence of HS^-. Lower panel. Chart record of the voltage trace containing the slow ramps (-110 to -40 mV). A2. Superimposed are the steady-state I-V curves showing they intersect at -90 mV, the E_{rev} of the HS^--mediated conductance increase. Inset A3. Digital subtraction of the curves in A2 showing the HS^--induced current. B1. Application of Cd^{2+} (200 μM) caused only a slight inward current. Co-application of HS^- in the presence of Cd^{2+} resulted in a complete abolition of the HS^--induced outward current with a further unmasking of a slight inward current. B2. Superimposed are the steady-state I-V relationships from B1. Inset B3. Digital subtraction of the curves in B2 showing the abolition of the HS^--induced current.


A 1
200 μM HS⁻
0 nA
100 pA
60 sec
-60 mV
2
3
HS⁻ effect in ACSF
Current (nA)
0.4
0.2
0.0
-0.2
-120
-100
-80
-60
-40
Voltage (mV)
0.25
0
-0.25
-0.5
Current (nA)
control
200 μM HS⁻
-110
-100
-90
-80
-70
-60
-50
-40
Voltage (mV)

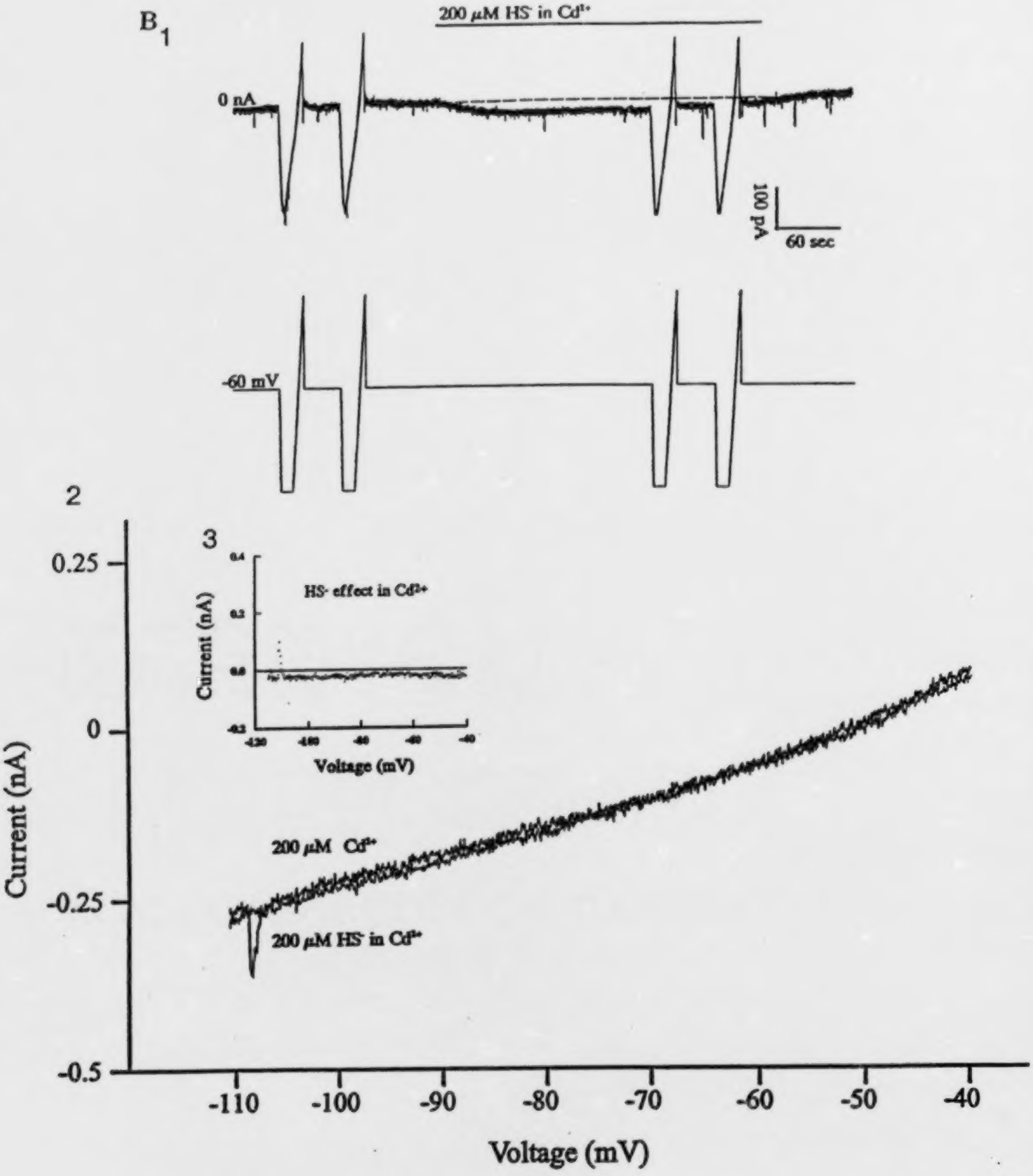
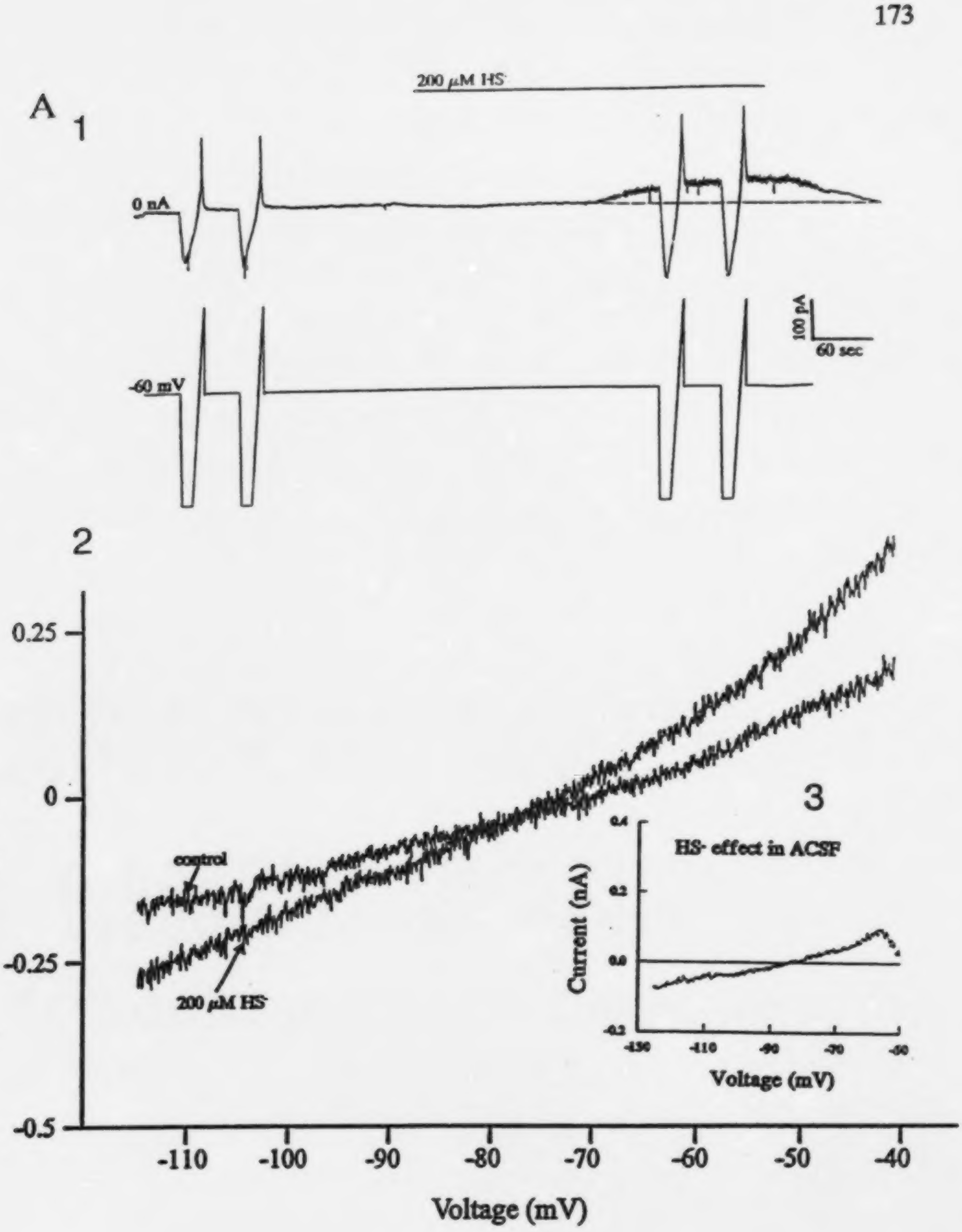
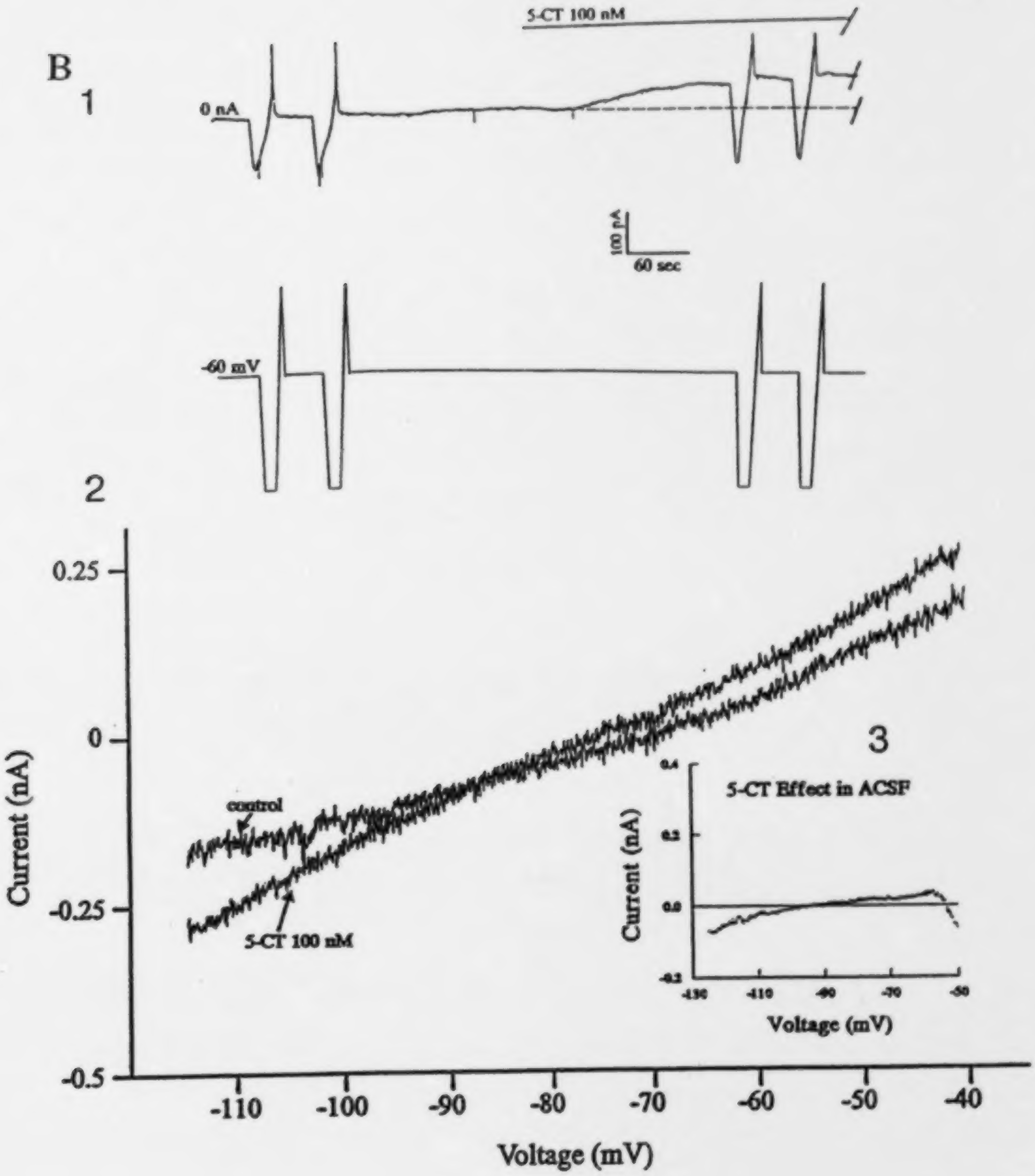
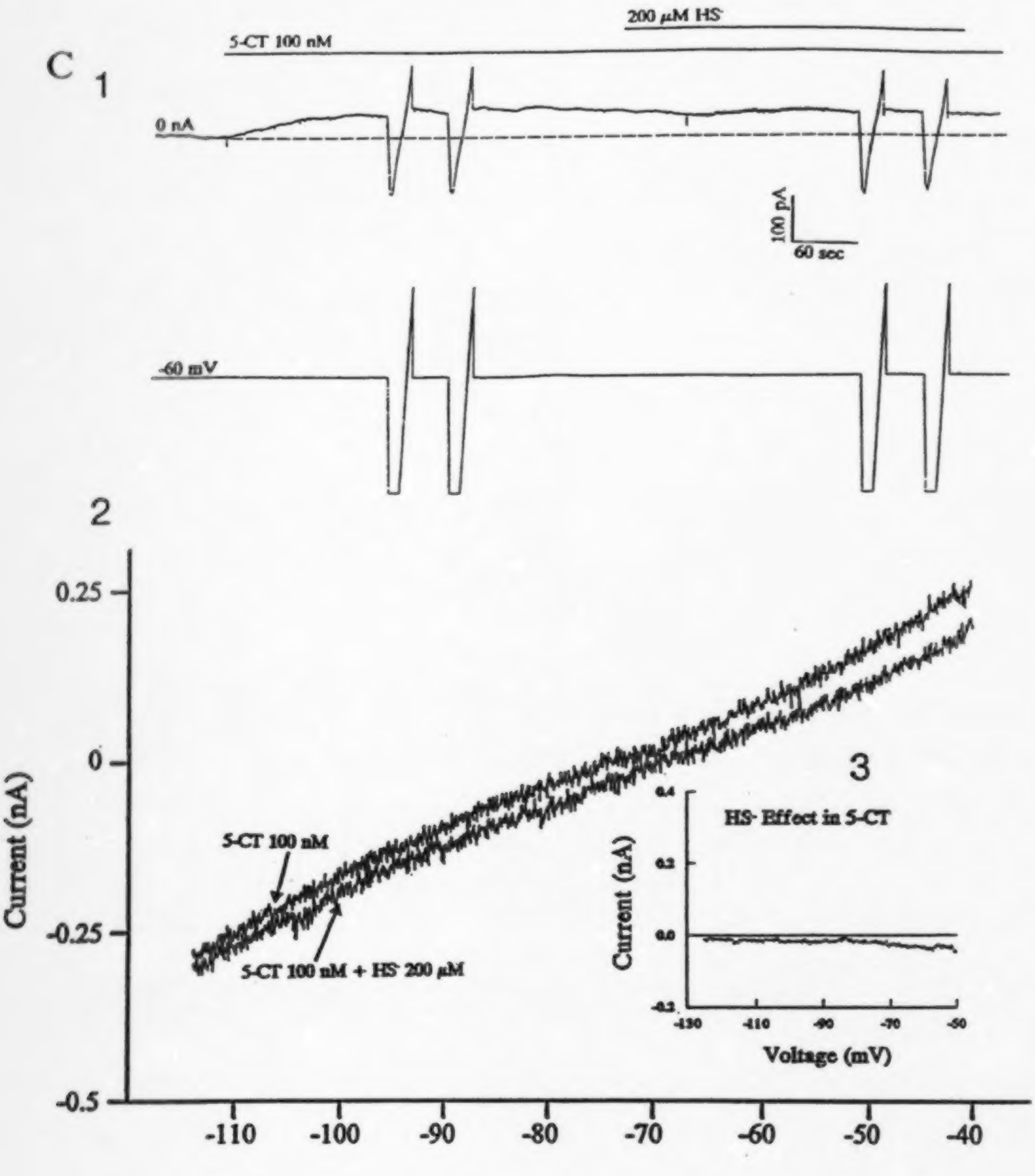

B1
200 μM HS- in Cd2+
0 nA
100 pA
60 sec
-60 mV
2
3
HS- effect in Cd2+
Current (nA)
0.4
0.2
0.0
-0.2
-120
-100
-80
-60
-40
Voltage (mV)
0.25
0
-0.25
-0.5
Current (nA)
200 μM Cd2+
200 μM HS- in Cd2+
-110
-100
-90
-80
-70
-60
-50
-40
Voltage (mV)

FIGURE 37. Effect of HS$^-$ on the 5-carboxamidotryptamine (5-CT) activated outward current in DR neurons. A1. 200 μM HS$^-$ induces an outward current that is accompanied by an increase in conductance. A2. Superimposed I-V relationships obtained from the slow voltage ramps (A1) applied in the absence (control) and presence of HS$^-$. Inset A3. Digital subtraction of the curves in A2 showing the HS$^-$ induced current and the E_{rev}. B1. 5-CT (100 nM) also induces an outward current with an increase in conductance, via activation of 5-HT$_{1A}$ receptors in the same neuron. B2. Superimposed I-V relationships from B1. Inset B3. Digital subtraction of the curves in B2 showing the 5-CT induced current and its E_{rev}. C1. Application of 200 μM HS$^-$ in the presence of 100 nM 5-CT on the cell above results in the occlusion of the HS$^-$ induced outward current and the unmasking of a small inward current. C2. Superimposed I-V relationships obtained in C1 and a digital subtraction of these curves to show the current induced by HS$^-$ in the presence of 5-CT (inset C3).


A
1
200 μM HS⁻
0 nA
100 pA
60 sec
-60 mV
2
0.25
0
-0.25
-0.5
Current (nA)
control
200 μM HS⁻
-110
-100
-90
-80
-70
-60
-50
-40
Voltage (mV)
3
HS⁻ effect in ACSF
0.4
0.2
0.0
-0.2
Current (nA)
-130
-110
-90
-70
-50
Voltage (mV)


B
1
5-CT 100 nM
0 nA
100 pA
60 sec
-60 mV
2
0.25
0
-0.25
-0.5
Current (nA)
control
5-CT 100 nM
-110
-100
-90
-80
-70
-60
-50
-40
Voltage (mV)
3
5-CT Effect in ACSF
0.4
0.2
0.0
-0.2
Current (nA)
-130
-110
-90
-70
-50
Voltage (mV)


C
1
5-CT 100 nM
200 μM HS-
0 nA
100 pA
60 sec
-60 mV
2
0.25
0
-0.25
-0.5
Current (nA)
5-CT 100 nM
5-CT 100 nM + HS- 200 μM
-110
-100
-90
-80
-70
-60
-50
-40
3
HS- Effect in 5-CT
0.4
0.2
0.0
-0.2
Current (nA)
-130
-110
-90
-70
-50
Voltage (mV)

treatment.

The sensitivity of the outward current to the removal of extracellular Ca^{2+} suggests the involvement of the latter in the HS^--induced response. As Ca^{2+} is generally known to modulate many channels (including K^+ channels) through surface charge interactions (see review by Rudy, 1988) in addition to the activation of Ca^{2+}-dependent channels, we were interested to know if the HS^- action was through the activation of Ca^{2+}-dependent channels (K^+ channels) or through surface charge interaction. To test the hypothesis that the HS^--induced outward current was through the activation of Ca^{2+}-dependent K^+ channels, we left the extracellular Ca^{2+} concentration intact and applied a Ca^{2+} channel blocker, limiting the entry of Ca^{2+} into the cell. Cadmium (Cd^{2+}) is a calcium channel blocker (Hirnig et al., 1988; Hille, 1991) which has been shown to eliminate Ca^{2+}-dependent K^+ currents in vagal motor neurons (Yarom et al., 1985). Application of 200 μM Cd^{2+} blocked the HS^- induced outward current and in addition, unmasked a very small inward current (Fig. 36B, n=4). Digital subtraction of the steady-state I-V curves revealed only the small inward current, with no outward current component, induced by HS^- over the entire voltage range (Fig. 36B3). The influx of extracellular Ca^{2+} into the cell may therefore account, at least in part, for the HS^--induced outward current.

In DR cells, 5-HT and its agonists act on $5\text{-}HT_{1A}$ autoreceptors to induce an inwardly rectifying K^+ current (Williams et al., 1988; Pan and Williams, 1989; Yoshimura and Higashi, 1985; Bobker and Williams, 1990). To test the hypothesis that HS^- activates such an inwardly rectifying K^+ current as part of its action, we

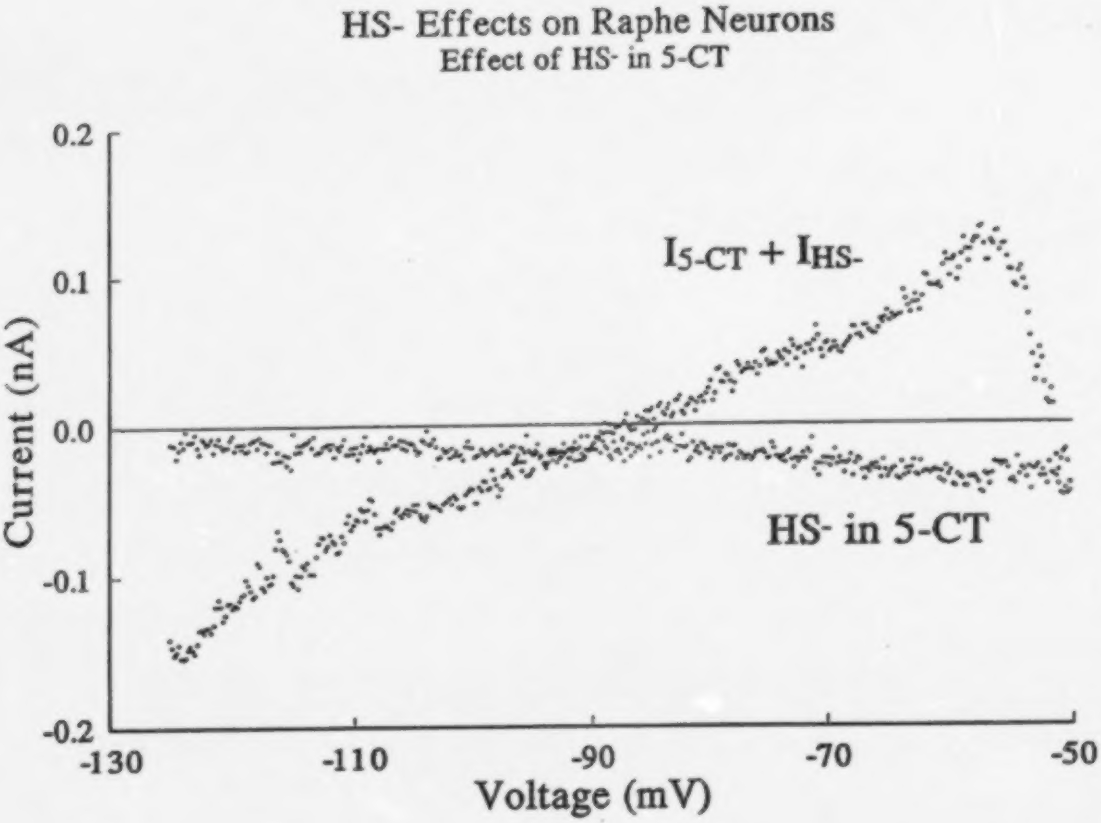


FIGURE 38. Digital addition of the 5-CT induced (Fig. 37B) and the HS$^-$ induced (Fig. 37A) outward currents and the HS$^-$ induced current in the presence of 5-CT.

applied HS^- in the presence of 5-CT, a 5-HT_1 agonist. Application of 200 μM HS^- induced an outward current that reversed at approximately -85 mV (Fig. 37A). Digital subtraction of the curve in control and that in the presence of HS^- revealed the current induced by HS^- (Fig. 37A inset). Application of 100 nM 5-CT alone produced an outward current that showed inward rectification and reversed at about -100 mV (Fig. 37B) Application of HS^- in the presence of 5-CT resulted in the blockade of the HS^- induced outward current with the unmasking of a small inward current (Fig. 37C; n=4). Digital addition of the HS^--induced and the 5-CT-induced outward currents yielded a curve with characteristics similar to that induced by 5-CT alone i.e inward rectification and an E_{rev} of about -90 mV (Fig. 38)

ii. Experiments to Determine the Mechanism(s) of the HS^--induced Inward Current.

In about 43% of cells studied, application of HS^- produced an inward current response (Fig. 39A). This response could be due either to an increased influx of Na^+ or Ca^{2+} ions which should be accompanied by an increase in conductance or a decrease in K^+ efflux out of the cell which should result in a decrease in conductance. To examine the ionic nature of this current, slow voltage ramps (-115 to -40 mV) were applied to these cells in the absence and presence of HS^-. Examination of the I-V curves revealed an inward shift (Fig. 39B). Digital subtraction of the two curves showed HS^- induced a weakly voltage dependent inward current (Fig. 39B inset). Removal of HS^- was always followed by a robust washout outward current (Fig. 39A; also see Fig. 29).

FIGURE 39. HS^- induces a weakly voltage-dependent inward current in some DR neurons. A. Upper panel. Chart record of the resting current of a DR neuron voltage clamped near rest (V_h= -60 mV) and the HS^--induced inward current. Two steady-state I-V relationships of the cell are taken at a minute interval by a slow voltage ramp protocol in control and in the presence of 200 μM HS^- (see Methods), averaged and stored. -//- 60 sec interruption in the chart record. Lower panel is the voltage trace showing the ramps. At the peak of the rebound outward current, 4 evenly spaced hyperpolarizing voltage steps, of 1 sec duration, were applied from V_h to increasingly negative potentials to determine the I-V relationship of this conductance. B1. The digitally averaged I-V relationships from A, in control and in 200 μM HS^- are superimposed. Inset B2. Digital subtraction of the I-V relationships in B1 revealing the HS^--mediated current.

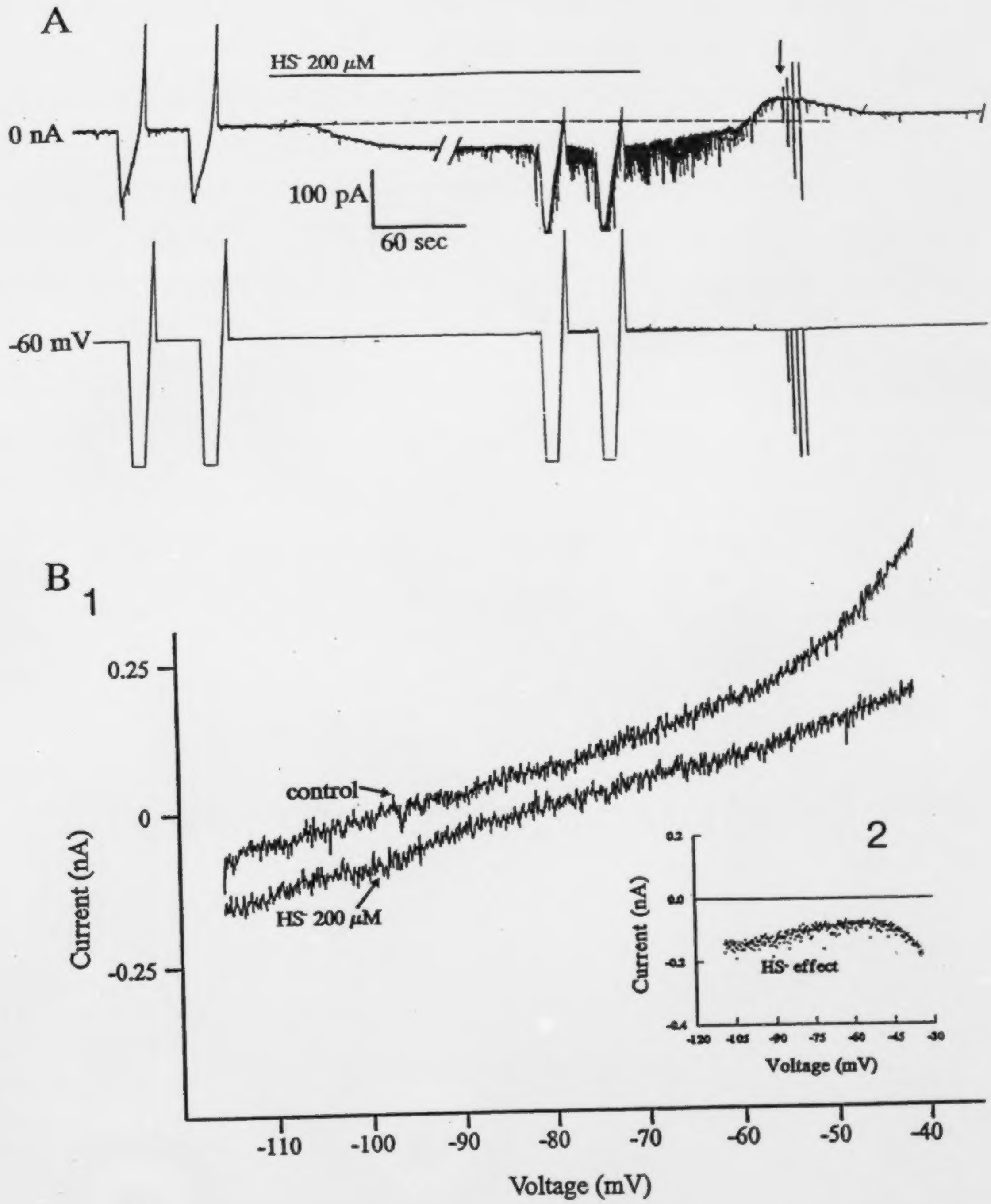

A
HS- 200 μM
0 nA
100 pA
60 sec
-60 mV
B
1
0.25
0
-0.25
Current (nA)
control
HS- 200 μM
-110
-100
-90
-80
-70
-60
-50
-40
Voltage (mV)
2
0.2
0.0
-0.2
-0.4
Current (nA)
HS- effect
-120
-105
-90
-75
-60
-45
-30
Voltage (mV)

FIGURE 40. Application of strophanthidin (7.5 μM) induces a non-saturating inward current in the cell in Fig. 39, with an I-V relationship identical to that induced by HS^- in Fig. 39. A. Chart record of current and voltage traces as in Fig. 39. -//- is a 6 min interruption in the chart record. B1. The I-V relationship obtained from A, in control (recovery from HS^- effect in Fig. 39) and in the presence of strophanthidin superimposed for comparison. Inset B2. Digital subtraction of the I-V curves in B1 showing the strophanthidin-induced current.

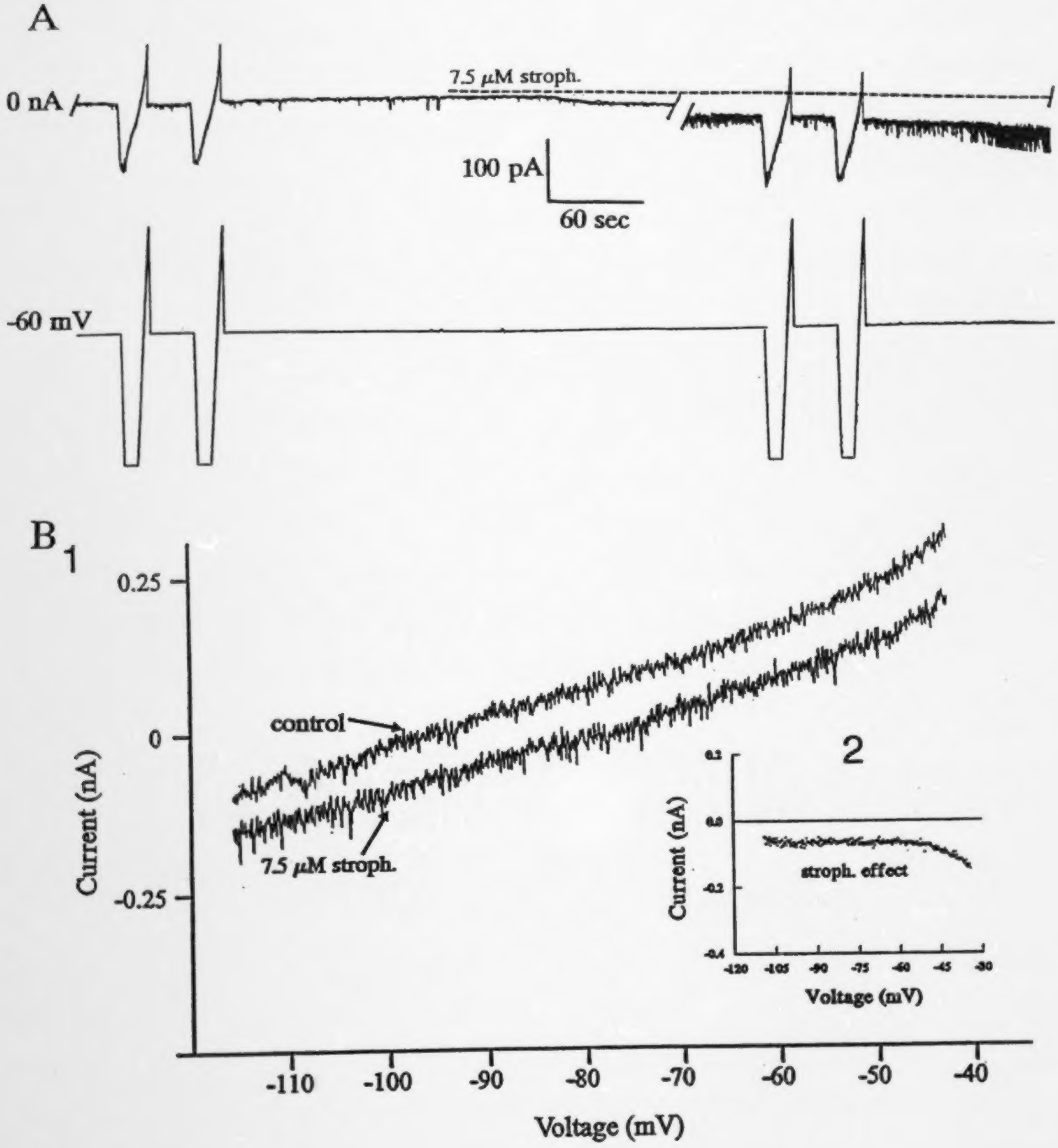
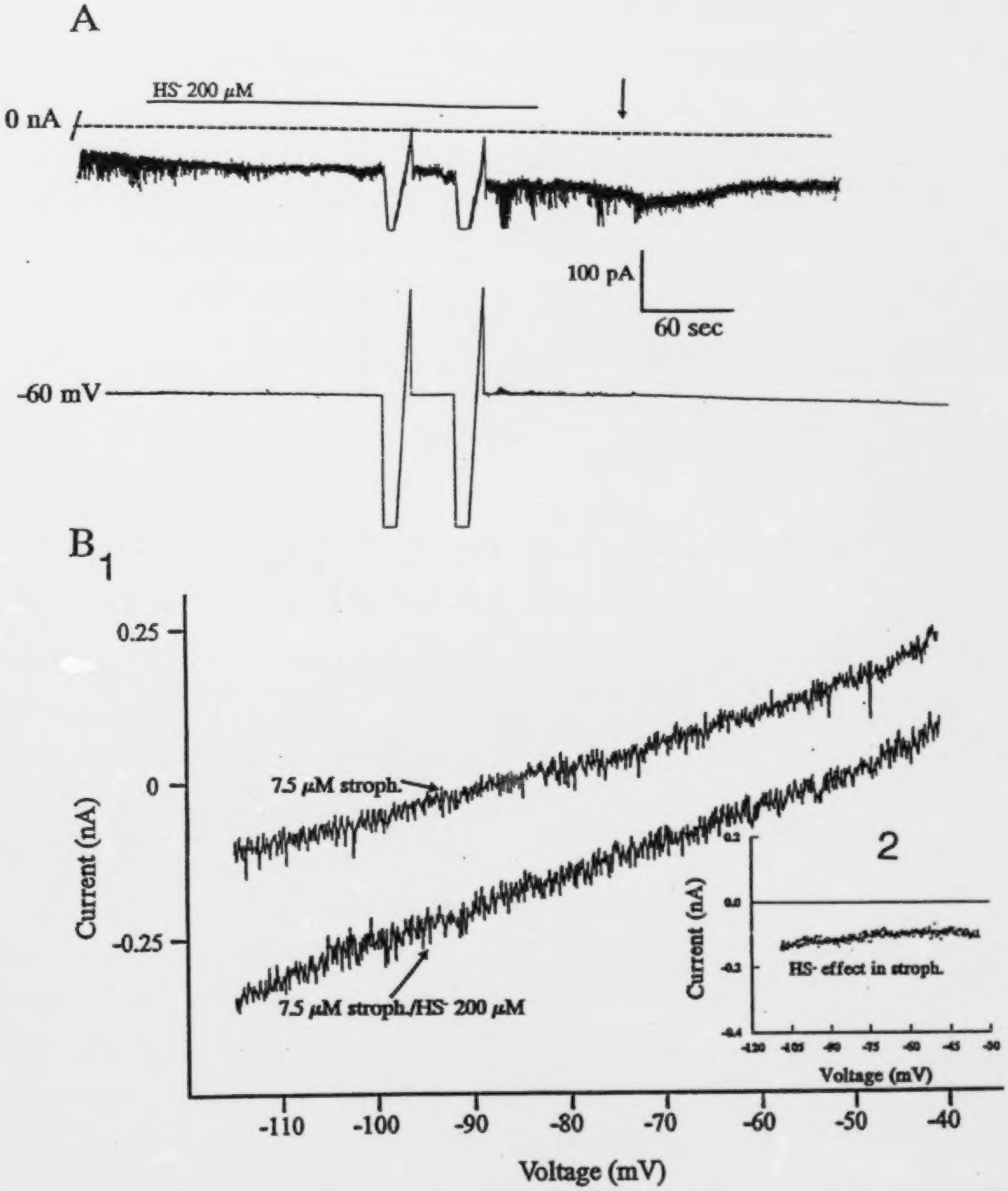
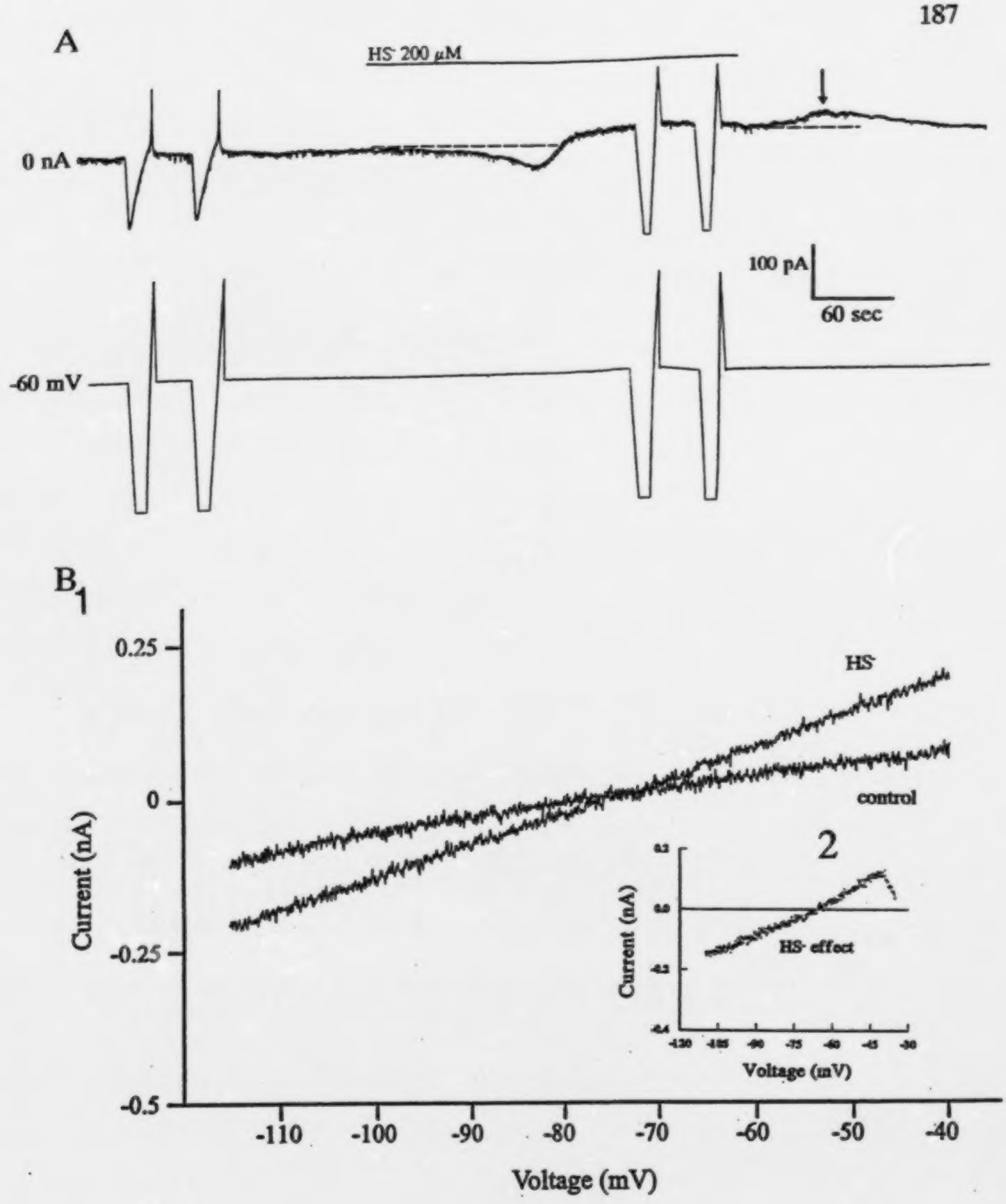

A
0 nA
7.5 μM stroph.
100 pA
60 sec
-60 mV
B 1
0.25
0
-0.25
Current (nA)
control
7.5 μM stroph.
-110
-100
-90
-80
-70
-60
-50
-40
Voltage (mV)
2
0.2
0.0
-0.2
-0.4
Current (nA)
stroph. effect
-120
-105
-90
-75
-60
-45
-30
Voltage (mV)

FIGURE 41. Effect of HS$^-$ (200 μM) in the presence of strophanthidin (7.5 μM) on the cell of Fig. 39. A. Chart record of the holding current and the voltage trace as described in Fig. 39. The inward current continued to increase as long as strophanthidin was present. Co-application of HS$^-$ (200 μM) further increased the inward current. Removal of HS$^-$ however, failed to elicit the characteristic washout outward current (see arrow). B.1. Superimposed are the steady-state I-V relationships in strophanthidin only (from Fig. 31) and in strophanthidin plus HS$^-$. Inset B2. Digital subtraction of the curves in B.1 to show the HS$^-$-induced current in the presence of strophanthidin.


A
HS- 200 μM
0 nA
100 pA
60 sec
-60 mV
B1
0.25
0
-0.25
Current (nA)
7.5 μM stroph.
7.5 μM stroph./HS- 200 μM
-110
-100
-90
-80
-70
-60
-50
-40
Voltage (mV)
2
0.2
0.0
-0.2
-0.4
Current (nA)
HS- effect in stroph.
-120
-105
-90
-75
-60
-45
-30
Voltage (mV)

The very weak voltage dependence and absence of an overt conductance change, together with the observed washout outward current, suggested that an inhibition of the electrogenic Na^+/K^+ ATPase pump may underlie this current. This pump current has been studied in several excitable tissues (Dean, 1964; Smith et al., 1986; see review by Phillis and Wu, 1981). The inhibition of the pump by removal of extracellular K^+ has been shown to lead to a depolarization while its reactivation with normal extracellular K^+ ion concentration produces a hyperpolarization (Rang and Ritchie, 1968; Phillis and Wu, 1981, Smith and Dombro, 1985; Smith et al., 1988). It has also been shown to be inhibited by cardiac glycosides such as ouabain and strophanthidin (Schatzmann, 1953; Skou, 1965; Fukuda and Prince, 1991). If HS^- inhibited a chronically active electrogenic pump, then a depolarization (inward current) will result. Therefore, to test the hypothesis that the HS^--induced inward current was due to inhibition of the pump, the latter was blocked with strophanthidin (5-7.5 μM) and the actions of HS^- examined. Application of strophanthidin produced an inward current (93.3 ± 8.8 pA; n=3) with an I-V relationship that is very similar to the one obtained in the presence of HS^- (Fig. 40). However, the strophanthidin effect did not attain a peak within the time frame of all experiments. For the above reason, the magnitude of the washout outward current was monitored as an index of pump inhibition. Application of HS^- in the presence of strophanthidin still induced an inward current at rest (86.7 ± 6.7 pA, measured from the level before HS^- application or 176.7 ± 17.6 pA measured from the level before strophanthidin application; n=3) but the removal of HS^- was not accompanied by a washout outward

FIGURE 42. Effect of HS^- on a DR neuron with a biphasic response to HS^-. A. Upper panel. Chart record of the membrane current and the steady-state current, produced in a DR neuron clamped near rest (V_h= -60 mV), by a slow voltage ramp protocol in control and in the presence of HS^- (200 µM). Application of HS^- first induces an inward current which is rapidly superceded by a sustained outward current. Removal of HS^- is followed 1-3 minutes later by the characteristic washout outward current (see arrow). Lower panel. The voltage trace with the ramp protocols. B1. Superimposed are the steady-state I-V relationships from A. The curves intersect at -70 mV, the E_{rev} of the HS^- mediated conductance increase. Inset B2. Digital subtraction of the curves in B1, showing the HS^--induced current. This current intersects the zero current line at equilibrium potential (E_{rev}) of the HS^--induced current. HS^- also appears to suppress or inhibit an outwardly rectifying conductance in the voltage range -50 to -40 mV.


A
HS⁻ 200 μM
0 nA
100 pA
60 sec
-60 mV
B1
0.25
0
-0.25
-0.5
Current (nA)
HS⁻
control
-110
-100
-90
-80
-70
-60
-50
-40
Voltage (mV)
2
0.2
0.0
-0.2
-0.4
Current (nA)
HS⁻ effect
-120
-105
-90
-75
-60
-45
-30
Voltage (mV)

current (Fig. 41) since ATPase was still inhibited by strophanthidin. Digital subtraction of the I-V curve in the presence of strophanthidin alone and in the presence of strophanthidin plus HS^- revealed that HS^- still induced an inward current even after the washout outward current was completely eliminated (Fig. 41B inset). The removal of strophanthidin was never followed by a washout outward current because the effect of strophanthidin was only slowly reversible. In one cell treated with strophanthidin, the application of HS^- after prolonged washing produced partial recovery of the washout outward current that usually follows the removal of HS^- (not shown).

iii. Experiments to Determine the Mechanism(s) of the HS^--induced Biphasic Responses.

A group of DR cells (≈ 30%) responded to sulfide with a biphasic response, first an inward current that was rapidly superceded by a sustained outward current that peaked in about 3-5 min from the time of application of HS^-. The washout of HS^- was often followed, in 1-3 min, by a washout outward current (Figs. 29 and 42A). To examine the nature of the currents underlying these different phases, they had to be studied in pharmacological isolation. However, the ionic characteristics of one phase had to be known in order to make an appropriate choice of antagonists to block it. Since the late outward phase achieved a steady-state peak in contrast to the early inward phase, the characteristics of this current were examined first. Slow voltage ramps (-115 to -40) were applied at the peak of the outward current to obtain steady-state I-V relationships. The curve obtained in the presence of HS^- (200 μM)

FIGURE 43. Effect of HS^- on the isolated inward current in the cell in Fig. 42. Application of barium (Ba^{2+}, 100 μM) and cesium (Cs^+, 2 mM) produced an inward current at -60 mV. A. Chart records (as described in Fig. 46) in the presence of Ba^{2+} and Cs^+ alone and in the latter two plus HS^- 200 μM. HS^-, in the presence of Ba^{2+} and Cs^+ produced a sustained inward current with complete blockade of the outward current component of the biphasic HS^--induced current. Removal of HS^- was then followed by a more robust washout outward current (arrow) than in control (Fig. 32). At the peak of this current, 4 evenly spaced voltage steps, of 1 sec duration, were applied from V_h to increasingly hyperpolarizing potentials to determine the I-V characteristics of this conductance. B1. The steady-state I-V relationship obtained from A are superimposed for comparison. Inset B2. Digital subtraction of the curves in B1, showing the HS^--induced current.

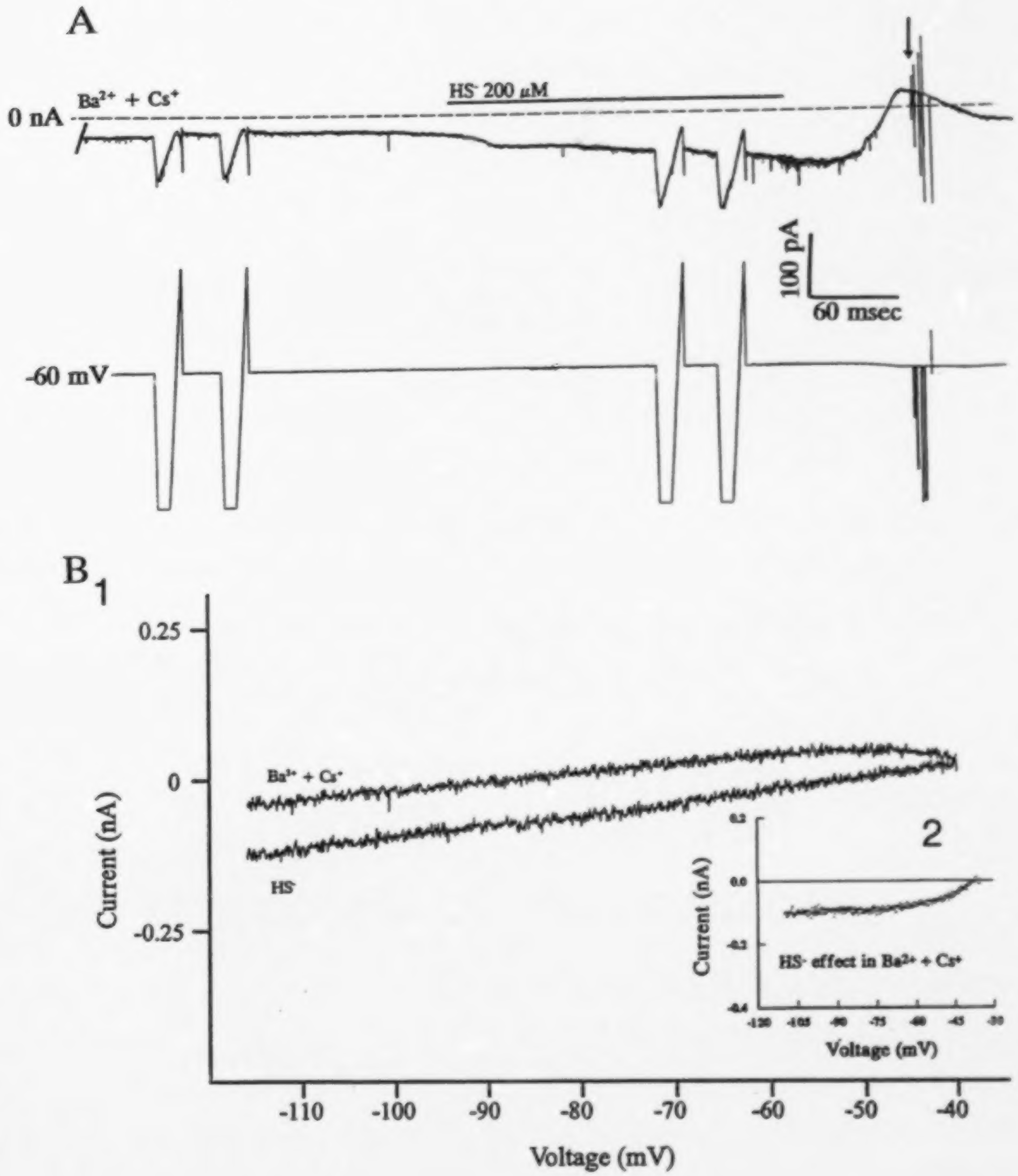
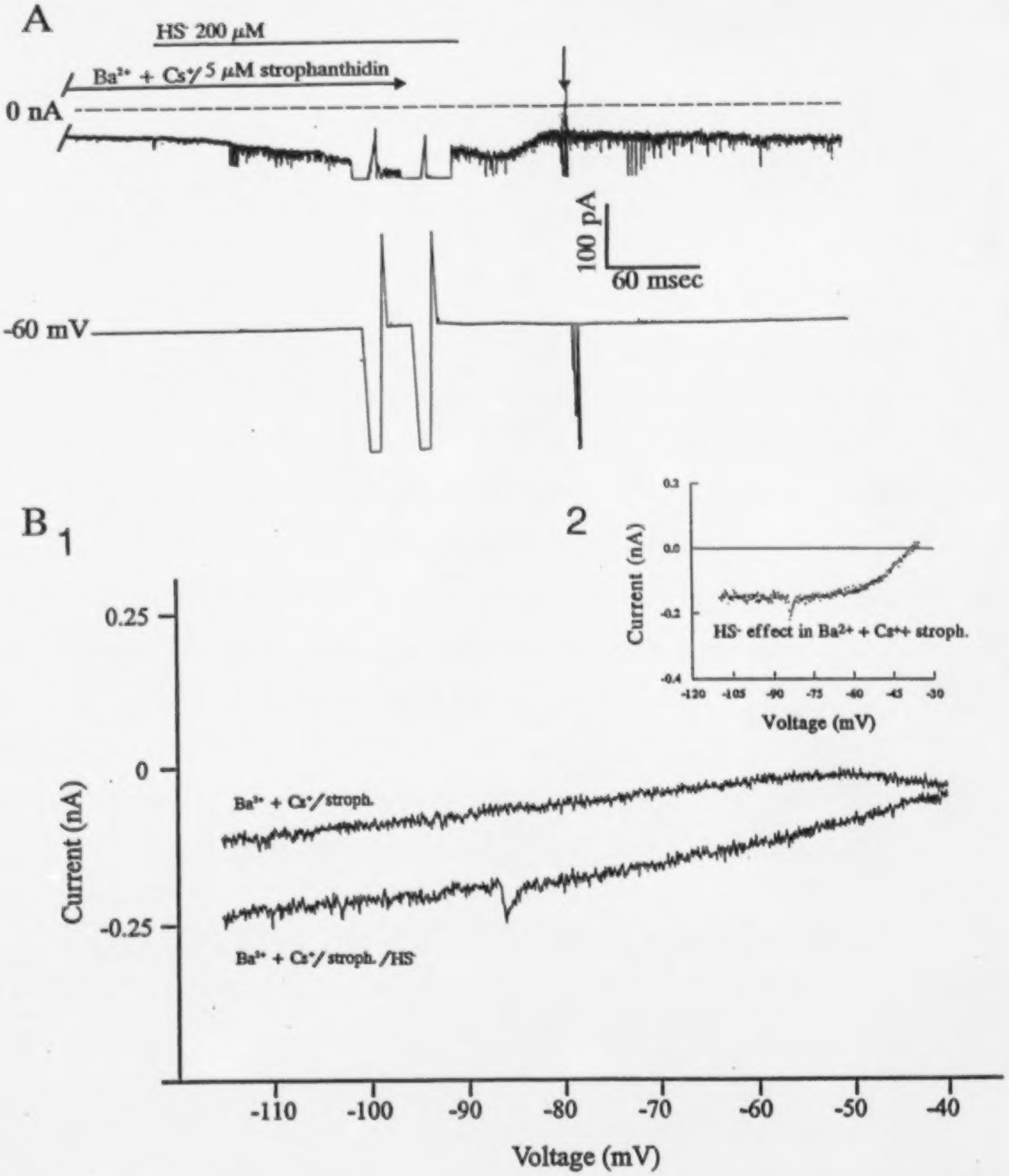

A
Ba2+ + Cs+
HS- 200 μM
0 nA
100 pA
60 msec
-60 mV
B1
0.25
0
-0.25
Current (nA)
Ba2+ + Cs+
HS-
-110
-100
-90
-80
-70
-60
-50
-40
Voltage (mV)
2
0.2
0.0
-0.2
-0.4
Current (nA)
HS- effect in Ba2+ + Cs+
-120
-105
-90
-75
-60
-45
-30
Voltage (mV)

FIGURE 44. Effect of HS^- on the same cell in Figs. 42 & 43 but in the presence of Ba^{2+}, Cs^+ and strophanthidin (5 μM). A. Chart records as described in Fig. 46. Application of 5 μM strophanthidin induces a further increase in inward current . HS^- application under this condition caused an increase in inward current but removal of HS^- now produces a much reduced washout outward current (see arrow). Prolonged washing (>45 min), in Ba^{2+} and Cs^+, produced a partial recovery of the outward current following HS^- removal (not shown). B1. Steady-state I-V curve in Ba^{2+}, Cs^+ and strophanthidin only (chart record not shown) and in the presence of all the above and HS^- for comparison. Inset B2. Digital subtraction of the steady-state curves in B1 showing the HS^--induced current under this condition.

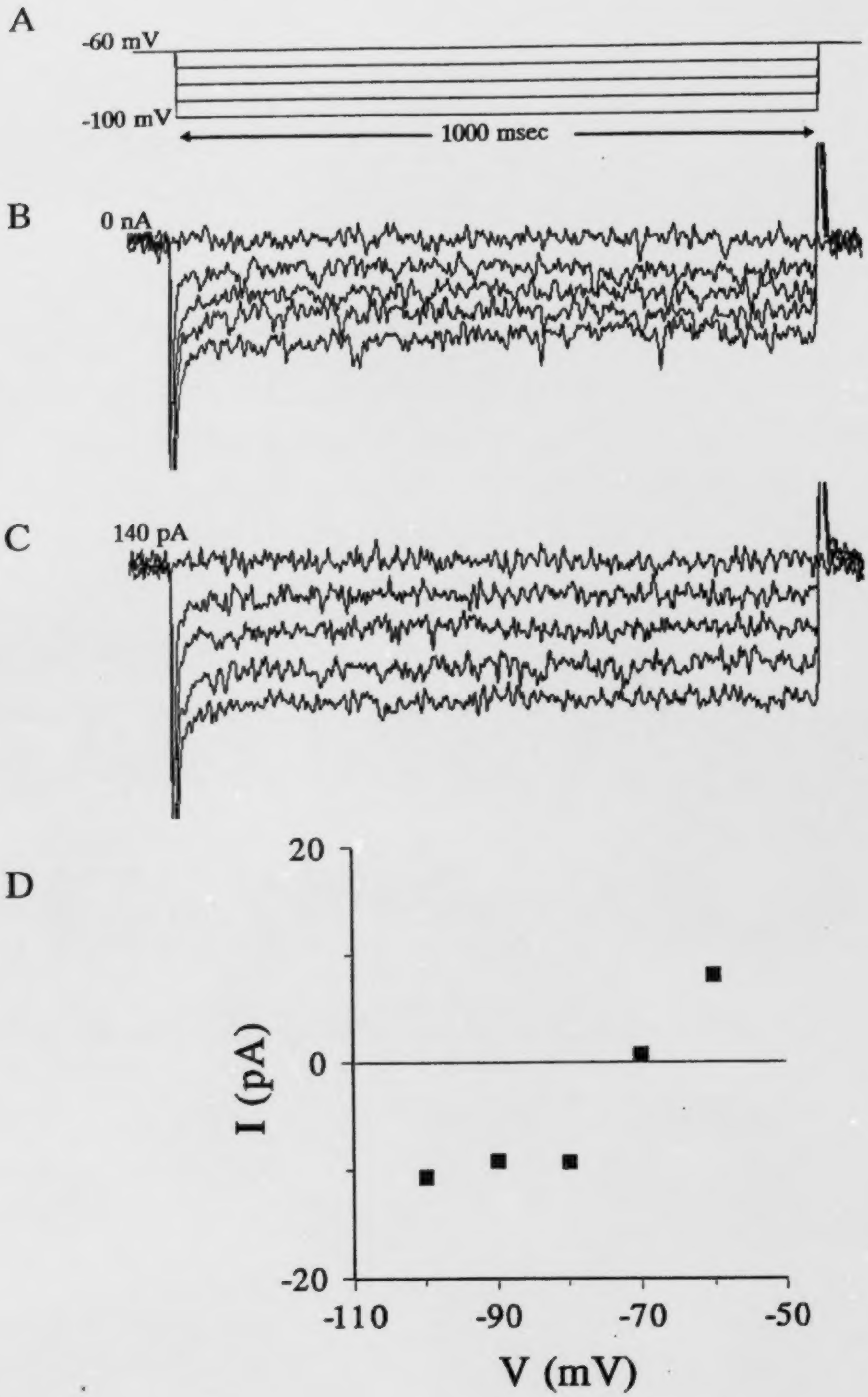

A
HS⁻ 200 μM
Ba²⁺ + Cs⁺/ 5 μM strophanthidin
0 nA
100 pA
60 msec
-60 mV
B 1
2
Current (nA)
0.2
0.0
-0.2
-0.4
-120
-105
-90
-75
-60
-45
-30
Voltage (mV)
HS⁻ effect in Ba²⁺ + Cs⁺+ stroph.
0.25
0
-0.25
Current (nA)
Ba²⁺ + Cs⁺/stroph.
Ba²⁺ + Cs⁺/ stroph. /HS⁻
-110
-100
-90
-80
-70
-60
-50
-40
Voltage (mV)

showed an increase in conductance over the control curve and intersected the latter at approximately -77.4 ± 1.9 mV (n=5; Fig. 42B). Digital subtraction of the control curve from the curve in the presence of HS^- revealed the HS^- induced current was very similar to that induced by HS^- in monophasic outward current cells (see Fig. 32). The equilibrium potential for the outward current induced by HS^- in biphasic cells was however positive (by about 10 mV) to that observed in monophasic outward current cells.

The qualitative similarity in the current induced by HS^- in monophasic outward current cells and these cells and the close identity of their E_{rev}s suggested to us that the outward phase in these cells was probably due to activation of a K^+ channel(s) identical to those in the monophasic outward current cells. To examine this fact, we tested the sensitivity of the outward current to blockade by a combination of Ba^{2+} and Cs^+. Application of these cations produced a small inward current. Subsequent application of HS^- in the presence of these K^+ channel blockers resulted in an enhanced inward current with no detectable outward current (n=3; Fig. 43A). The washout of HS^- under these condition resulted in a robust washout outward current (Fig. 43A). Examination of the steady-state I-V relationships obtained in the presence of Ba^{2+} and Cs^+ and in the presence of these cations and HS^- revealed only an inward shift in the HS^--induced current with little or no change in conductance (Fig. 43B; n=3). Digital subtraction of these curves reveal the isolated inward current was very similar to that recorded in monophasic inward current responders (Fig. 43B inset).

To examine if this inward current was also due, in part, to inhibition of the

FIGURE 45. The effect of changing membrane potential on the washout outward current. A. At resting membrane current and at the peak of the washout outward current in fig. 39, hyperpolarizing voltage steps of 1 sec duration were applied, 10 sec apart, from V_h (-60 mV) to V_c at 10 mV increments. B. The corresponding currents evoked by the voltage steps in A applied in control (rest). C. Currents corresponding to the voltage steps in A applied at the peak of the washout outward current. D. A plot of the difference current after digital subtraction of the steady-state currents in B from that in C.


A
-60 mV
-100 mV
1000 msec
B
0 nA
C
140 pA
D
20
0
-20
I (pA)
-110
-90
-70
-50
V (mV)

Na^+/K^+ ATPase, the pump was inhibited by strophanthidin (5 μM, submaximal concentration) in the presence of Ba^{2+} and Cs^+ and the actions of HS^- tested in the same cell. Application of HS^- in the presence of all of the above (Ba^{2+}, Cs^+ and strophanthidin) still caused an inward current, but the removal of HS^- was now followed by a much reduced washout outward current (~20% of control; n=2; Fig. 44A). The I-V curves obtained during these treatments were similar to those obtained in monophasic inward current cells treated with strophanthidin (Fig. 44B)

iv. Experiments to Determine the Nature of the Washout Outward Current.

In approximately 90% of all responding DR cells, the washout of HS^- was always followed, in 1-3 min, by a washout outward current of about 155.8 ± 18.3 pA (n=12). In current clamp recordings, this washout effect was seen as a hyperpolarization. The washout outward current was shown to be blocked only by strophanthidin, a Na^+/K^+ ATPase pump inhibitor, but not Ba^{2+} and Cs^+, K^+ channel blockers. This suggested the involvement of an electrogenic pump current in the response. To determine the electrical characteristics of this current, 1 sec duration hyperpolarizing voltage steps were applied from V_H, in 10 mV increments, at the peak of this current and the corresponding currents recorded (Fig. 45A & B). Similar voltage steps were applied at resting conductance for comparison. Digital subtraction of the steady-state currents in control from those obtained at the peak of the washout outward current yielded the washout current at various voltages (Fig. 45C). Although the magnitude of the washout outward current was approximately 160 pA, the current

FIGURE 46. In about 13% of all the DR neurons examined in this study, HS^- did not evoke a significant postsynaptic response. A. Upper panel. Chart records of the resting current of a DR neuron clamped at -60 mV (near rest) and the steady-state currents obtained from the ramp protocols (lower panel) used to obtain them, in control, and then in the presence of 200 μM HS^-. Lower panel shows the voltage trace and the ramp protocols. B1. Superimposed are the steady-state I-V relationships in control and in the presence of HS^- showing the lack of effect of HS^- on the membrane conductance of this cell over the voltage range examined. Inset B2. Digital subtraction of the curves in B1 showing the lack of effect of HS^- over a large voltage range.

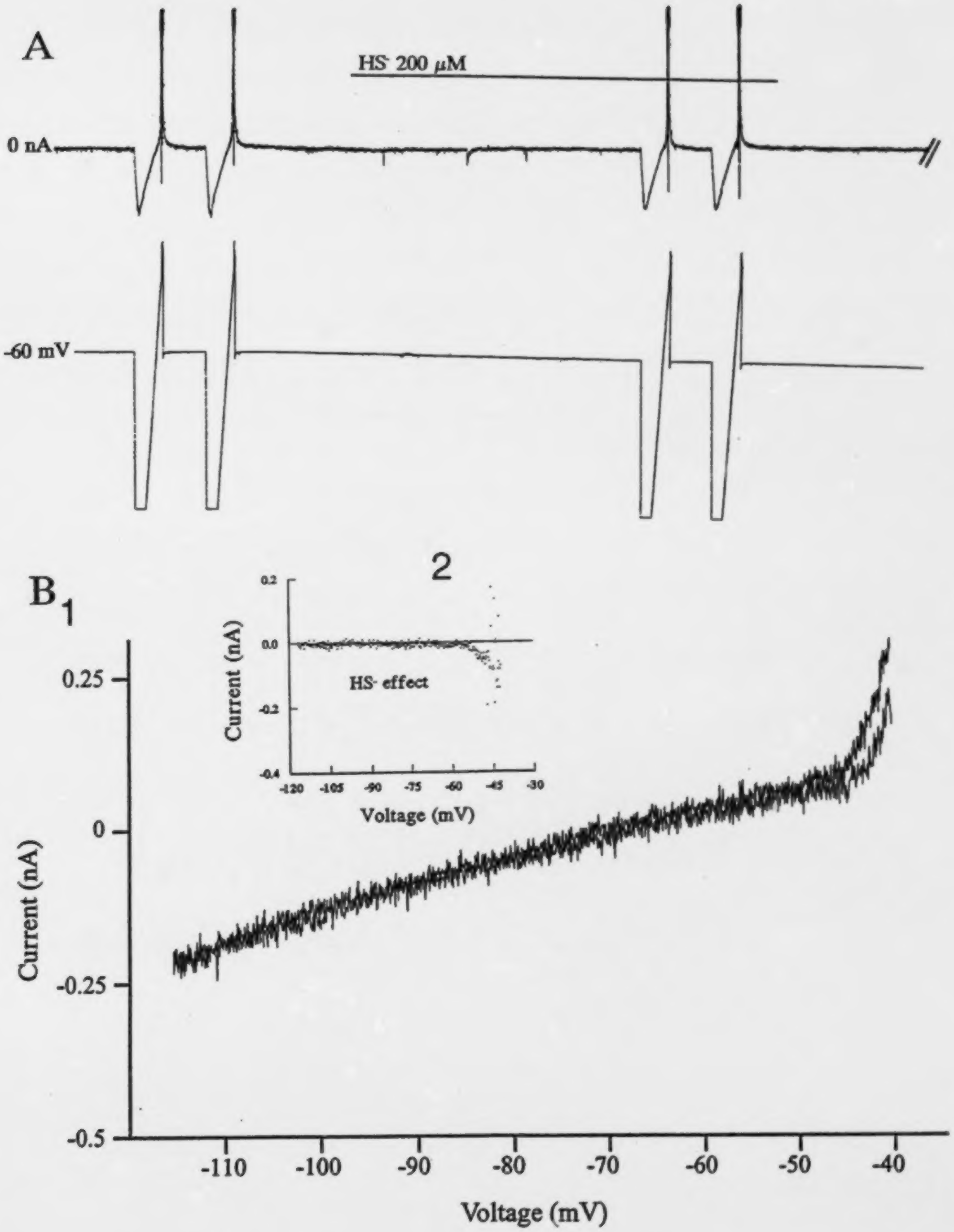
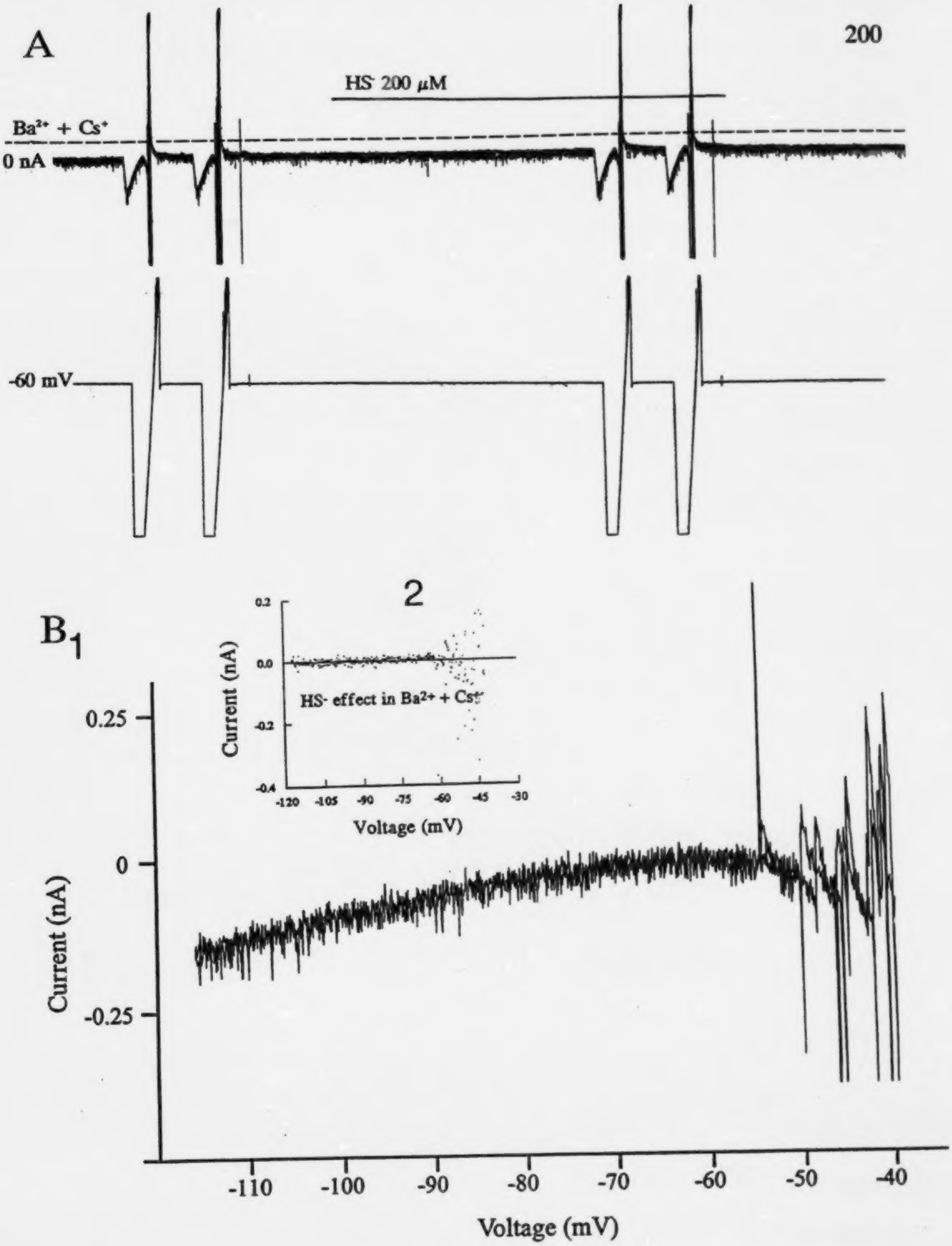

A
HS- 200 μM
0 nA
-60 mV
B
1
2
0.2
0.0
-0.2
-0.4
Current (nA)
HS- effect
-120
-105
-90
-75
-60
-45
-30
Voltage (mV)
0.25
0
-0.25
-0.5
Current (nA)
-110
-100
-90
-80
-70
-60
-50
-40
Voltage (mV)

FIGURE 47. Ba^{2+} and Cs^{+} do not alter the response of the cell in Fig. 46 to HS^{-}. A. Application of Ba^{2+} and Cs^{+} induced an inward current in DR neurons (dotted line represents the resting current prior to the addition of Ba^{2+} and Cs^{+}). Chart records showing the resting current in the presence of Ba^{2+} and Cs^{+} and the steady-state currents obtained by the voltage clamp protocols as shown in the lower panel. Unclamped action potentials occurred at the more depolarized regions of the I-V curves under both conditions. Current data at voltages positive to -55 mV is therefore unreliable. B. Superimposed are the steady-state I-V curves from A, showing that Ba^{2+} and Cs^{+} did not alter the response of the cell to HS^{-} over the entire voltage range examined. Inset B2. Digital subtraction of the curves in B1, again showing the lack of effect of HS^{-} on postsynaptic conductances of these cells in the presence of Ba^{2+} and Cs^{+}.


A
HS⁻ 200 μM
Ba²⁺ + Cs⁺
0 nA
-60 mV
B1
2
0.2
0.0
-0.2
-0.4
Current (nA)
HS- effect in Ba²⁺ + Cs⁺
-120
-105
-90
-75
-60
-45
-30
Voltage (mV)
0.25
0
-0.25
Current (nA)
-110
-100
-90
-80
-70
-60
-50
-40
Voltage (mV)

difference from control even at the highest command voltage was only about 20 pA indicating the voltage-independence of this current.

v. Experiments to Examine the Electrophysiological Characteristics of the Non - Responsive Cells.

Approximately 13% of cells in the DR were non-responsive to toxicologically relevant doses of HS^- ($\geq$ 200 μM). In these cells, application of 200 μM HS^- produced no change in membrane currents at rest (Fig. 46). To examine if these cells, which met all the criteria of serotonergic DR cells (See Methods; Williams et al., 1988), had any voltage dependent conductances that HS^- may affect in a particular voltage range, we applied slow voltage ramps (-115 to -40 mV) in the absence and presence of HS^-. Comparison of the I-V curves in control to those in the presence of HS^- revealed no difference in the curves over the entire voltage range (n=9; Fig. 46B). Digital subtraction of these curves confirmed HS^- activated no detectable current over the voltage range examined in these cells (Fig. 46B inset).

Since we had observed both inward and outward current responses to HS^- and in some cases a combination of both in the same cell, we reasoned that HS^--insensitive cells may express both inward and outward currents but which were equal in magnitude and with identical latency of activation by HS^-. Under such a circumstance, one would expect a net zero change in resting current in response to HS^-. If this were the case, then complete elimination of one component should unmask the other. Since we had earlier demonstrated that the outward current induced by HS^- was blocked by a combination of Ba^{2+} and Cs^+, we applied these cations to determine if we could unmask an inward current. The application of HS^- in the presence of these cations did not produce any change in resting current nor did it change the I-V relationship of these cells over the voltage range studied (n=4; Fig. 47). This was confirmed by digital subtraction of the curves in the absence and presence of HS^- that revealed no current was induced by HS^- (Fig. 47B inset).

B. EFFECTS OF SULFIDE ON SYNAPTIC RESPONSES IN THE DR NUCLEUS.

As synaptic responses enable individual neurons to perform their integrative functions and to initiate action potentials that are important in cell to cell communication (Kuffler et al., 1984), perturbation of these responses can lead to altered neuronal signalling. To determine if HS^- produced its toxic effects by perturbing synaptic transmission, the effects of increasing concentrations of HS^- were examined on the synaptic responses in DR neurons *in vitro*. Focal electrical stimulation of DR neurons elicits a triphasic complex synaptic response (Pan and Williams, 1989; Yoshimura and Higashi, 1985; Williams et al., 1988; see Methods) that can be recorded either in current clamp or voltage clamp. As in the earlier studies in DR, we used toxicologically relevant concentrations of HS^-, concentrations in the range between 30 μM and 1000 μM were applied.

In current clamp experiments, focal electrical stimulation in or near the DR elicited complex synaptic potentials (rmp -55 to -70 mV; Fig. 48). There was an initial

FIGURE 48. HS^- inhibits synaptic responses in the DR nucleus. Upon impalement, most DR neurons rest at between -60 and -70 mV in current clamp. A. A focal electrical stimulus (20-50 V intensity and 100-400 msec duration) elicits a complex, triphasic synaptic response. An initial amino acid-mediated fast depolarizing synaptic potential (DSP) lasting between 20-30 msec, is followed by a 5-HT_{1A} autoreceptor mediated inhibitory postsynaptic potential (IPSP), lasting about 700 to 1000 msec,which in turn is followed by a longer lasting slow depolarizing postsynaptic potential (sEPSP). The clock was slowed 8-times to capture the decay of the sEPSP and the return of the cell to rest. B. Superimposed are synaptic responses elicited, in a different cell, in control and in the presence of HS^- (200 μM) showing the inhibition of synaptic responses. Both responses were elicited at the same resting potential, by the injection of appropriate current during HS^- application, to return the cell to the original resting membrane potential. C. Superimposed are the synaptic responses in HS^- (from B) and the response following 10-15 min wash in ACSF (recovery) demonstrating that the effects of HS^- on synaptic responses in the DR are reversible.

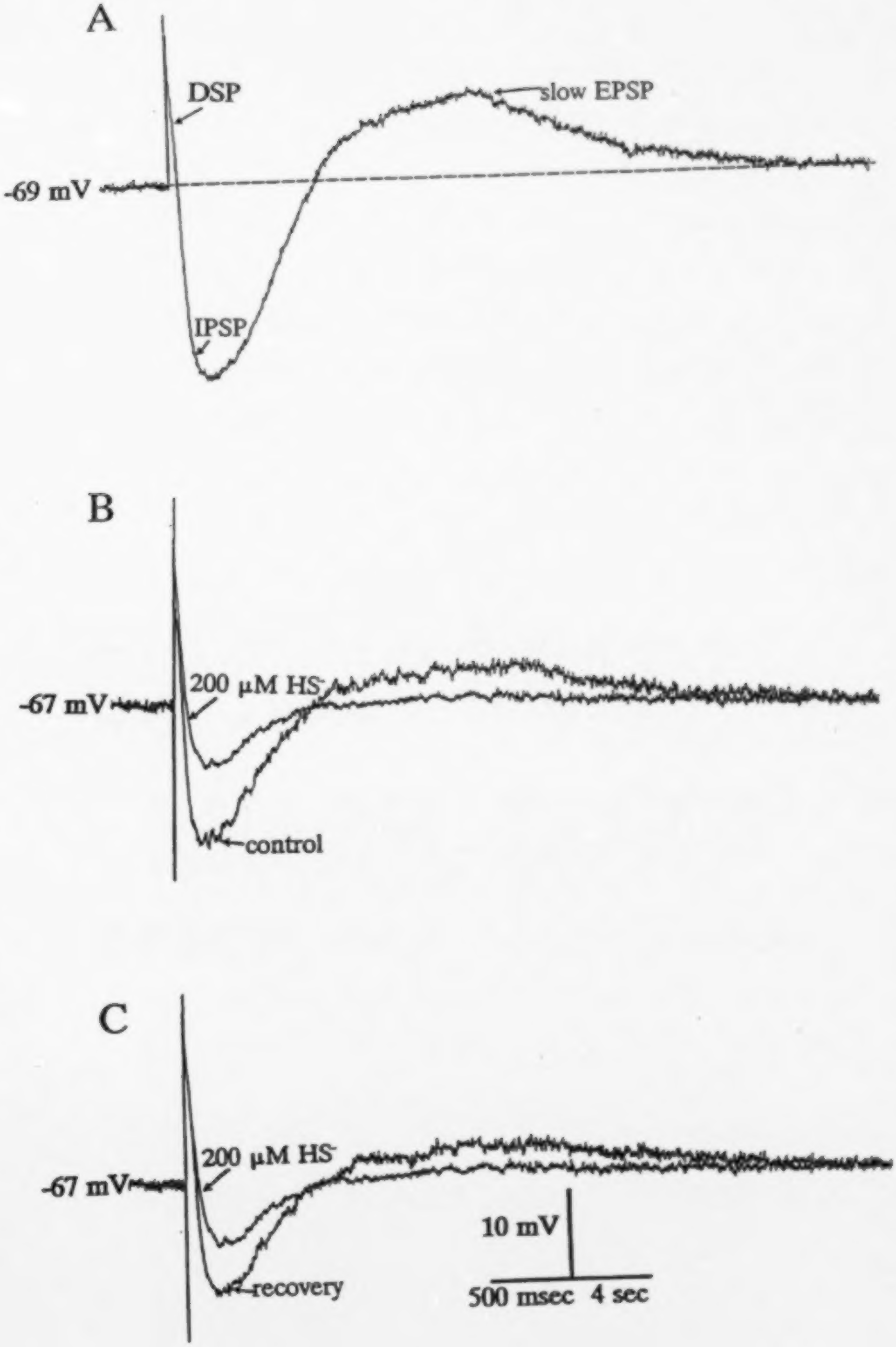
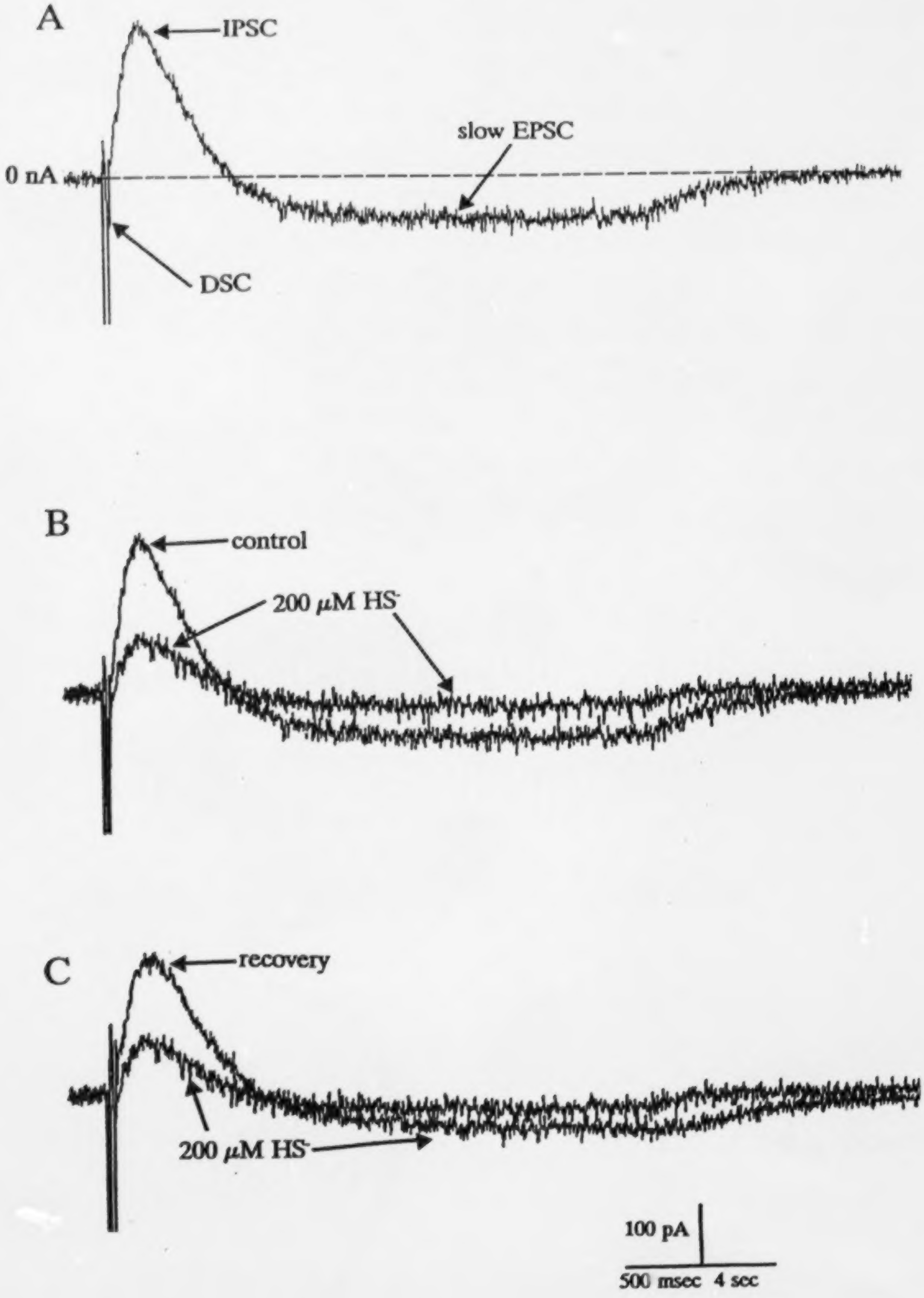

A
DSP
slow EPSP
-69 mV
IPSP
B
200 μM HS-
-67 mV
control
C
200 μM HS-
-67 mV
recovery
10 mV
500 msec
4 sec

FIGURE 49. When an impaled DR neuron is voltage clamped, the currents underlying the synaptic potentials recorded in current clamp in Fig. 48 can be recorded (A). HS^- also reversibly inhibits these currents (B & C). All other details as in Fig. 37.


A
IPSC
slow EPSC
0 nA
DSC
B
control
200 μM HS$^-$
C
recovery
200 μM HS$^-$
100 pA
500 msec 4 sec

depolarizing postsynaptic potential (DSP) that was followed by an inhibitory postsynaptic potential (IPSP). Although there is a $GABA_A$ component to the DSP, the Cl^- used in the electrode dialysed the cell, altering this component to a depolarizing response. The IPSP is then succeeded by a slow excitatory postsynaptic potential (slow EPSP; Fig. 48A). Three to four minutes application of HS^- produces a peak postsynaptic response. Elicitation of synaptic responses at the peak of HS^--induced postsynaptic response demonstrated a concentration-dependent inhibition of all the components of the complex synaptic potential (Fig. 51). Fig. 48B shows the effect of 200 μM HS^- on the synaptic potentials superimposed on the potentials in control. At this concentration of HS^-, the DSP was reduced by about 72%, the IPSP by 71. 95 ± 6.9% and the slow EPSP by 79. 45 ± 7.6% ($p< 0.001$, $n=5$, Fig. 48B). This inhibition was reversible upon washout (10-15 min with ACSF, Fig. 48C)

If the impaled cells are held near rest in voltage clamp, the currents that underlie the synaptic potentials above can be recorded (Fig. 49A). Inhibition of these currents by HS^- can also be demonstrated as illustrated by the response in the presence of 200 μM HS^- (Fig. 49B), recovery occurring within 10-15 min of HS^- washout (Fig. 49C) The inhibition and recovery of the fast depolarizing response can be clearly observed on an expanded time scale (Fig. 50A & B).

The application of a range of concentrations of HS^- (30- 1000 μM) produced a concentration-dependent inhibition of all three components of the complex synaptic response. Fig. 51 shows the concentration-response curve constructed for the inhibition of the synaptic currents (DSC, IPSC and slow EPSC) by HS^-.

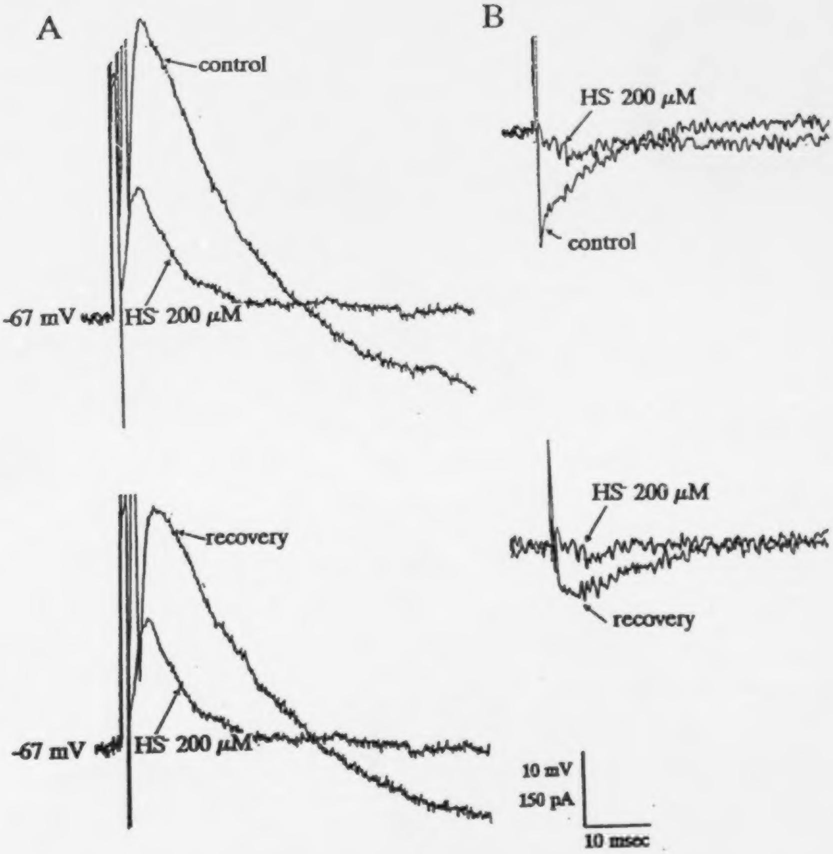


FIGURE 50. Because the initial synaptic response (DSP/DSC) in the DR neuron are very fast events, it is difficult to see if HS⁻ affected them. An expansion of the time scale in the responses in Fig. 47 & 48 allows better examination of the DSP (a) and DSC (b). HS⁻ reversibly inhibits these amino acid mediated responses.

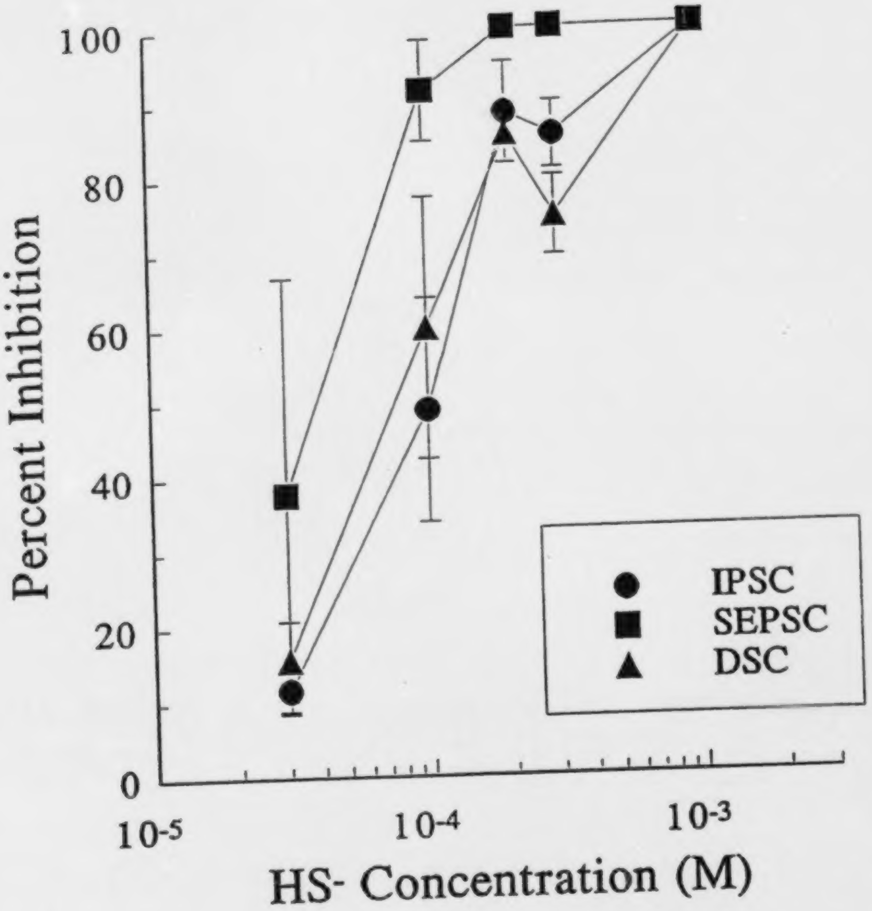


FIGURE 51. The inhibition of synaptic responses in the DR nucleus by HS^- is concentration dependent. Concentration-response curves of HS^- on the DSC, IPSC and sEPSC. HS^- inhibits the DSC (▲) with an EC_{50} of approximately 83 μM, the IPSC (●) with an EC_{50} of about 103 μM and the sEPSC (■) by about 45 μM.

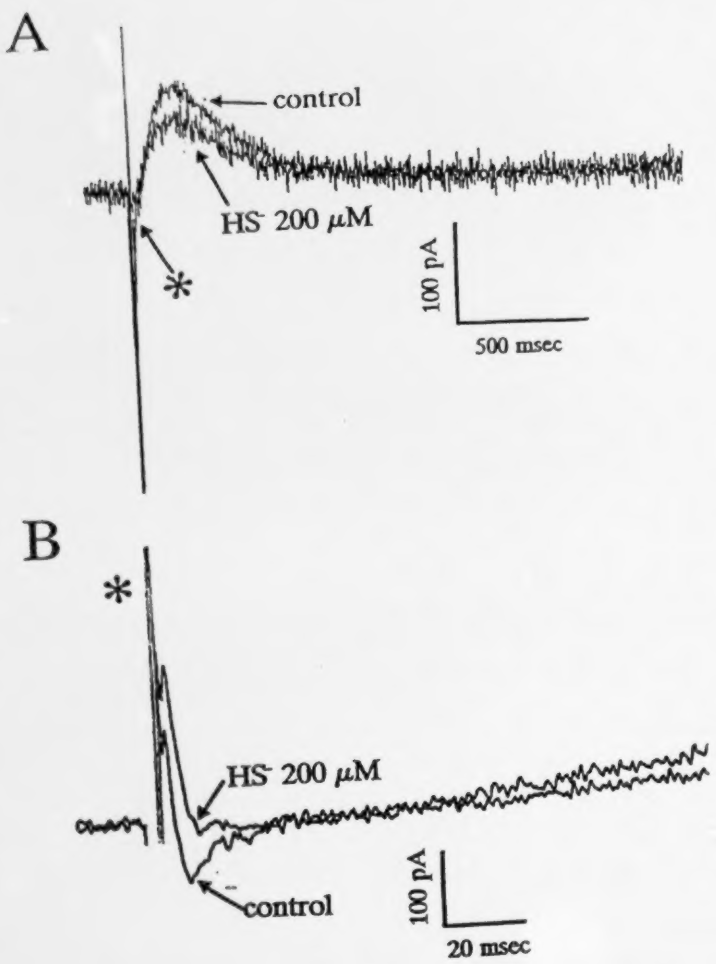


FIGURE 52. Although HS⁻ does not affect the postsynaptic membrane conductances of non responding cells, it slightly inhibits the IPSC and DSC recorded in these neurons. A. superimposed are synaptic responses evoked in the cell in Fig. 46 in control and in the presence of HS⁻ (200 μM). 200 μM HS⁻ inhibits the IPSC by 23.6 ± 4.94% (compared to the ≥ 80% reduction in responding cells) with a much more substantial reduction in the DSC. B. the asterick in A represents the DSC which has been recorded on an expanded time scale to more clearly show the effect of HS⁻ on this response.

Concentrations below 30 μM did not produce measurable changes in any of the synaptic currents while concentrations of 300 μM and above completely eliminated all synaptic responses. Fig. 49 shows the control synaptic current superimposed on the current in the presence of 200 μM HS^-. This dose of HS^- inhibited the DSC by 85.7 ± 3.9% ($p< 0.05$, $n=4$; Fig. 49), the IPSC by 88.5 ± 6.8% ($p< 0.05$, $n=4$; Fig. 49), while completely abolishing the slow EPSC ($n=4$; Fig. 38).

The ED_{50} values of the HS^- inhibition of all the synaptic responses were estimated from the concentration-response curves to be $\approx$83 μM for the DSC; $\approx$103 μM for the IPSC; and $\approx$45 μM for the slow EPSC (Fig. 51). 200 μM HS^-, used for studies of HS^- on postsynaptic membrane properties produced approximately 70-80% reduction in all the synaptic responses.

Synaptic currents recorded in DR neurons with no postsynaptic response to HS^- (nonresponders), revealed a reduction in the IPSC by 23.6 ± 4.9% of control ($p< 0.05$, $n=3$) in the presence of 200 μM HS^-. The DSC was completely abolished (Fig. 52)

V. *IN VIVO* TOXICITY STUDIES.

a. Toxicity Studies of Dithiothreitol (DTT) in Rats.

Based on the known chemistry and biochemical reactions of DTT, (protection of thiol and disulfide groups; Cleland, 1963; Jocelyn, 1987) and the HS^- ion (formation of persulfides; Wood, 1987), Warenycia et al., (1990) examined the ability of DTT to alter the HS^- inhibition of monoamine oxidase (MAO) enzyme and showed that DTT could reverse the inhibition of MAO by HS^- both *in vitro* and *ex vivo*. In preliminary *in vivo* studies, it was observed that DTT pretreatment prolonged the survival time of rats exposed to lethal levels of HS^- (Warenycia et al., 1988 and 1990). At this point, the toxicity profile of DTT in rats was unknown (LD_{50} value was only available for mice, 169 mg/kg by IP injection; Sax and Lewis, 1985). I therefore examined the toxicity profile of this agent in rats in order to determine the optimal dose that would protect or reverse sulfide effects in poisoned victims without producing toxic effects itself.

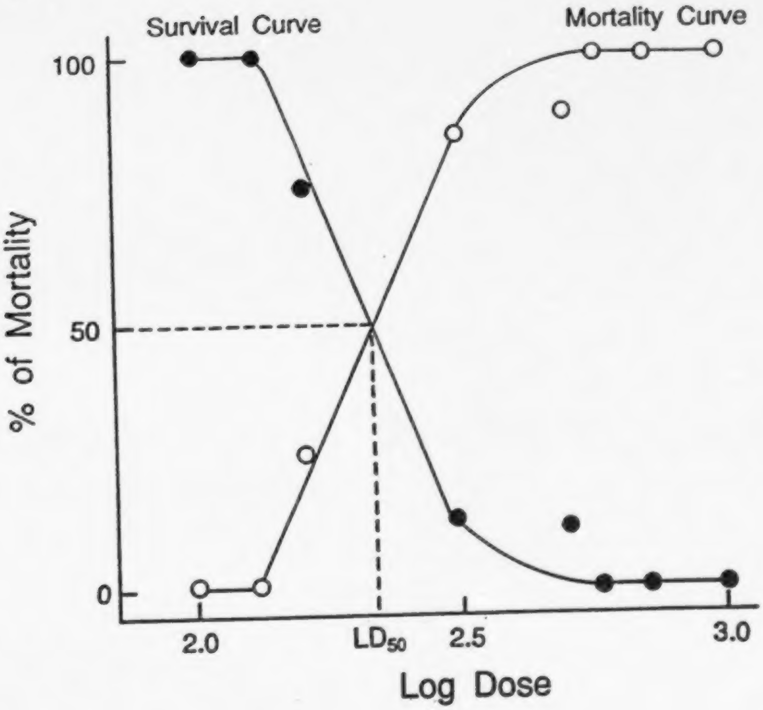
Doses of 100 to 1000 mg/kg of DTT were administered to rats by the IP route and cumulative percent mortality and survival was determined by the method of Read and Munch (see Methods). Graphs of percent mortality and survival versus $\log_{10}$ concentration of DTT were plotted. These curves intersected at a point which corresponded approximately, to the LD_{50} of DTT. This value was approximately log 2.33 which converted to an LD_{50} value of 214 mg/kg (Fig. 53A). Alternatively, both curves could be fitted to a line of best fit by linear regression. The intersection of the percent mortality line on the abscissa corresponded to the LD_0 value of 79.4 mg/kg (Fig. 53B). The LD_{50} value estimated by this method was 199.5 mg/kg (Fig. 53B). The estimated LD_1 value (81.3 mg/kg) was then employed in all protective studies.

b. Studies on the Ability of DTT to Protect HS^- Poisoned Rats.

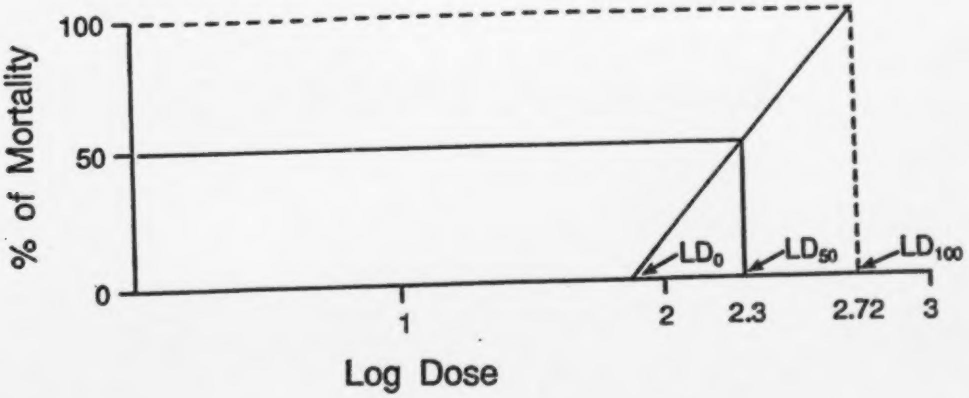
Previous toxicity studies of hydrogen sulfide using NaHS as the source of the toxic ion (HS^-), determined the concentration-mortality curve of sulfide and the LD_{50}

FIGURE 53. Concentration-response curves of dithiothreitol (DTT) in Sprague-Dawley rats following intraperitoneal injection. A. Concentration-mortality curve superimposed on the concentration-survival curve. The LD_{50} can be determined from both curves as the point of intersection of these curves correspond to the concentration where mortality= survival (50%). Data points were accumulated from groups containing at least 5 animals, and was calculated by the method of Reed and Muench (Turner, 1965). B. Linear regression of the mortality curve enabled the estimation of the LD_0 (79.4 mg/kg) and the LD_{50} (199.5 mg/kg). The LD_1 was then estimated by interpolation from the LD_0 and LD_{50} values to be 81.3 mg/kg. This concentration was used in all protective and antidotal studies.

A


Survival Curve
Mortality Curve
100
50
0
% of Mortality
2.0
LD$_{50}$
2.5
3.0
Log Dose

B


100
50
0
% of Mortality
LD$_0$
LD$_{50}$
LD$_{100}$
1
2
2.3
2.72
3
Log Dose

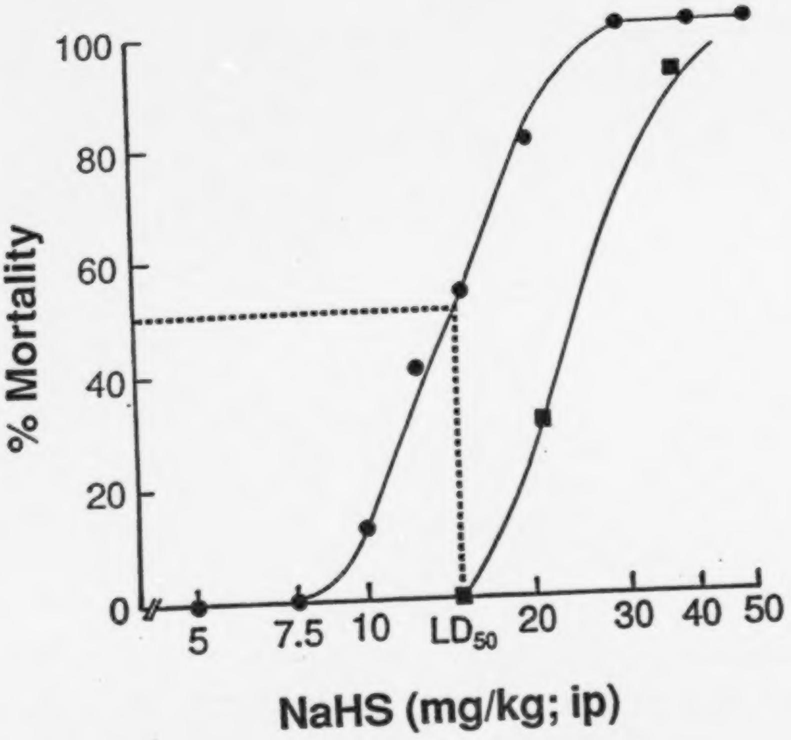


FIGURE 54. Protection against HS^- lethality by DTT. Superimposed are the control sulfide-lethality curve (● ; taken from Warenycia et al., 1989) and the curve after pretreatment with DTT (■). DTT-treated animals received 81.3 mg/kg (LD_1) IP, 20 min before HS^-; N=8 at each HS^- dose. The dose-response curve was then constructed from data calculated by the cumulative method of Reed and Muench (see Methods). The LD_{50} for HS^- after DTT pretreatment shifted to 25 mg/kg.

EFFECT OF DTT TREATMENT ON SULFIDE POISONING IN RATS

DOSE OF SULFIDE (MG/KG)	PERCENT MORTALITY AT TIME:-		
	0 SEC	30 SEC	60 SEC
15	50	28.60*	50
20	87	85.70	80
30	100 $C_n = 16$	100 $C_n = 17$	92.86 $C_n = 16$

C_n = Cumulative Total Number of Rats

* Significant decrease in mortality

TABLE 1 The success of treating HS^- poisoning with dithiothreitol (DTT) is dependent on both the magnitude of HS^- exposure and the time lapse between exposure and the administration of DTT. Whereas a substantial degree of survival (43% survival rate as compared to control values) was achieved at the low dose of 15 mg/kg HS^-, if DTT was given 30 sec afterwards, no significant changes in survival was observed at higher HS^- concentrations and more than 30 sec after HS^- poisoning.

was estimated to be approximately 15 mg/kg, with 30 mg/kg producing 100% mortality (Warenycia et al., 1988). In this study, 3 doses of sulfide, (15, 20 and 30 mg/kg) were administered to carefully randomized groups of rats (≥ 5 per group) all of which had 20 min earlier, received IP injections of 81.3 mg/kg DTT. The concentration-mortality curve of HS^- following pretreatment with DTT was clearly shifted to the right when compared to the curve without DTT pretreatment (Fig. 54). The estimated LD_{50} for HS^- in the latter experiment was approximately 25 mg/kg.

c. Studies on the Antidotal Potential of DTT.

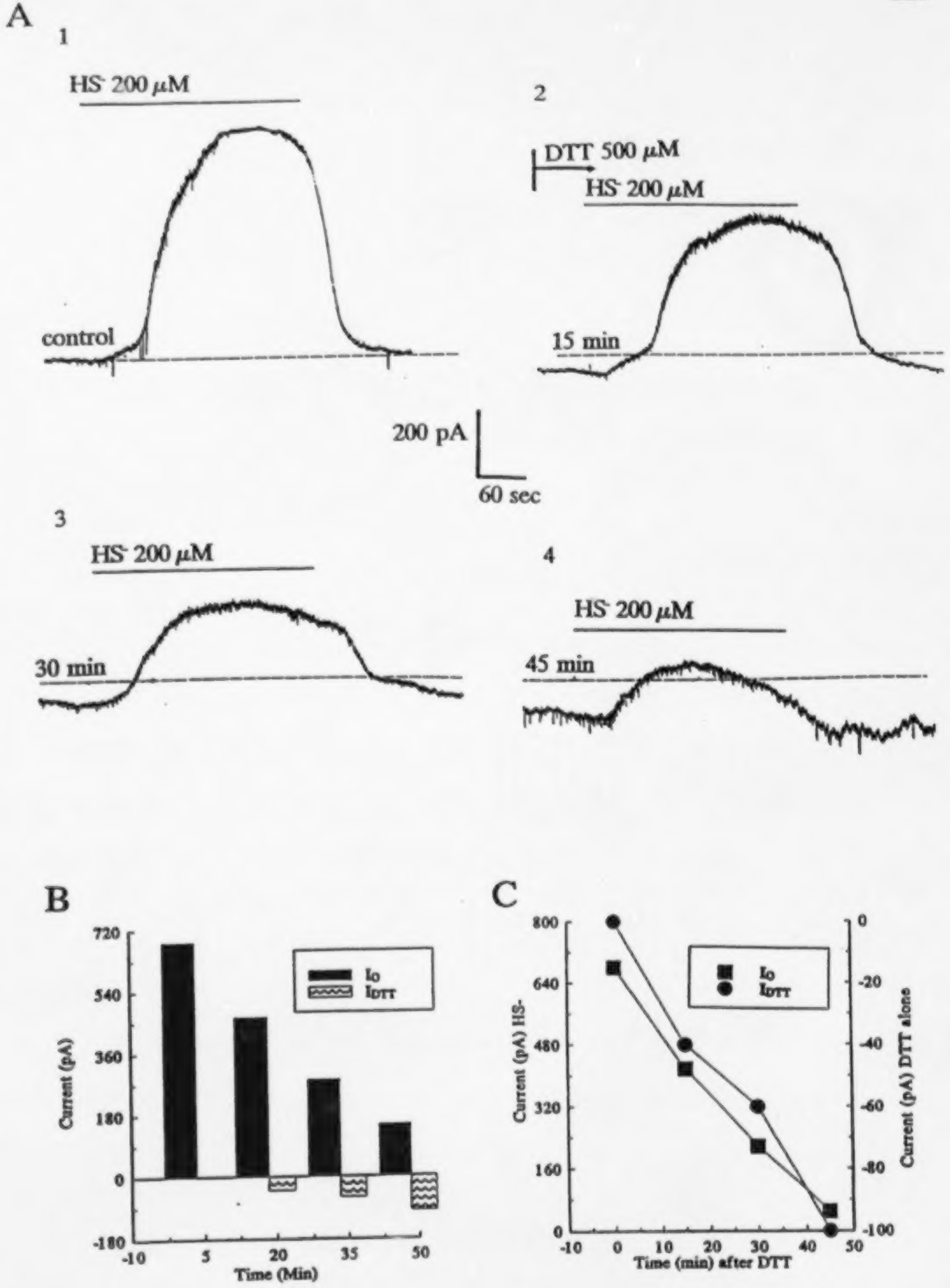
In order for DTT to be useful clinically, it should be able to reverse the toxic effects of sulfide following exposure. Pretreatment with DTT is not practicable as it is itself toxic and will require frequent dosing in anticipation of an exposure. To examine if DTT could be a potential antidote to sulfide poisoning, I investigated the ability of DTT to reverse effects of sulfide following lethal doses in rats. DTT was therefore given at different times after sulfide exposure. The timing of DTT administration was crucial as sulfide acts very rapidly (death occurring within 2 min of lethal exposure). DTT was therefore administered IP 30 and 60 sec after IP injection of sulfide. Mortality decreased to 57% of control mortality levels (43% survival) at only 15 mg/kg dose after of HS^-. This observed improvement in survival occurred only when DTT treatment was commenced 30 sec after poisoning with HS^-. No protection was detected at higher doses of HS^- and more than 30 sec exposure to HS^- prior to the commencement of treatment with DTT (Table 1).

VI. STUDIES ON *IN VITRO* PROTECTIVE EFFECTS OF DTT ON SULFIDE ACTIONS.

In vivo protective studies with DTT showed the ability of this compound to protect rats against HS^- poisoning (increasing the LD_{50} value of HS^- almost two-fold). However, the ability of DTT to reverse the toxicity of HS^- was not good, especially at higher exposure levels and if administered more than 30 sec after HS^- exposure (Table 1). I therefore wanted to determine if there were electrophysiological correlates of these *in vivo* observations, in addition to the known biochemical actions (Warenycia et al., 1989 and 1990).

To test the protective action of DTT on neurons, 527 μM DTT (equivalent to 81.3 mg/kg or LD_1) was applied to DR neurons held in voltage clamp near rest (V_h= -60 mV) and HS^- (200 μM) was applied at various time intervals after the application of DTT (Fig. 55A & B, n=3). DTT itself induced an inward current that increased with time throughout the experiment. A time-dependent inhibition was observed in the HS^--induced outward current (Fig. 55A & B). This effect of DTT on the HS^- induced current was steeply concentration-dependent, as a reduction of 50% in the concentration of DTT (263 μM; n=2) was ineffective in reducing the HS^--induced current (not shown). A correlation analysis of the DTT-induced inward current versus the DTT-mediated decrease in HS^--induced outward current showed them to be highly correlated (R= 0.99, Fig. 55C). This would suggest that part of the reduction in the HS^--induced current is due to the opposing inward current produced by DTT. However, a comparison of the magnitude of the decrease of the HS^--induced current

FIGURE 55. Dithiothreitol (DTT) inhibits the HS^--induced outward current in DR neurons following pretreatment in a time dependent manner. A. Application of DTT (527 μM, equivalent to the LD_1 in rats) induced an inward current. Co-application of HS^- (200 μM) 15 min after DTT resulted in about 40% reduction in the magnitude HS^--induced outward current. Prolonged application of DTT resulted in a continuous increase in the inward current and a much pronounced reduction in the HS^--induced outward current. B. A histogram summarizing the time-dependent inhibition of the HS^--induced outward current by DTT (■, rate of decrease = 0.73) and also the time-dependent increase in the DTT-induced inward current (▨, rate of increase = 0.18). C. A correlation graph comparing the DTT-induced inward current with the HS^--induced outward current in the presence of DTT. The correlation coefficient (R) was determined to be 0.99.


A
1
HS⁻ 200 μM
control
2
DTT 500 μM
HS⁻ 200 μM
15 min
200 pA
60 sec
3
HS⁻ 200 μM
30 min
4
HS⁻ 200 μM
45 min
B
Current (pA)
720
540
360
180
0
-180
-10
5
20
35
50
Time (Min)
I_O
I_DTT
C
Current (pA) HS-
800
640
480
320
160
0
-10
0
10
20
30
40
50
Time (min) after DTT
Current (pA) DTT alone
0
-20
-40
-60
-80
-100
I_O
I_DTT

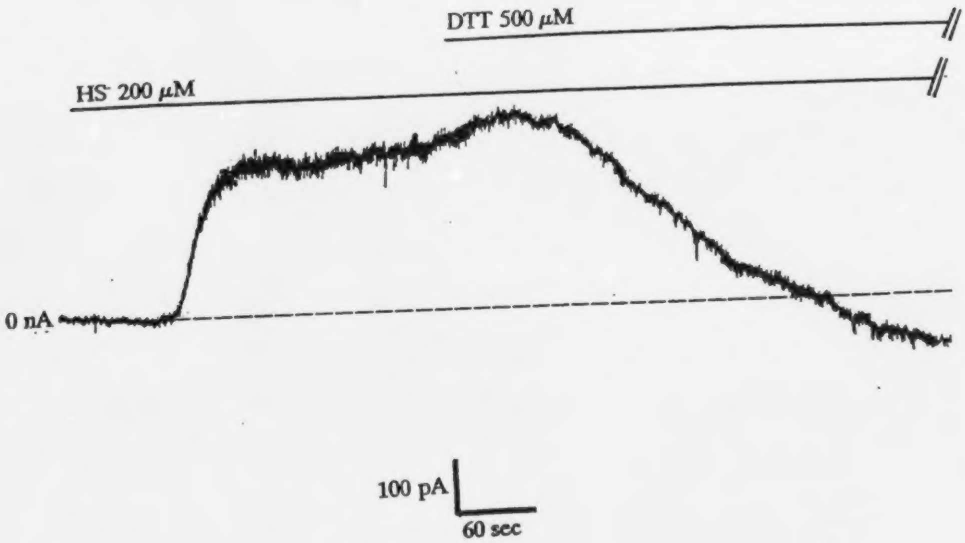


FIGURE 56. DTT slowly reverses the HS$^-$-induced outward current in a DR neuron voltage clamped near rest (V_h= -60 mV). Application of HS$^-$ (200 μM) induced an outward current which peaked in about 3 min. It was left on for an additional 2 min to ensure that the response did not decay or desensitize back to rest. DTT (500 μM) was then applied and it slowly (over a period of 4 min) reversed the HS$^-$-induced response to rest and beyond (inducing an inward current).

in the presence of DTT with time (slope= 0.73) to the magnitude of increase in the DTT-induced inward current with time (slope= 0.18), as in Fig. 55B, shows a greater than 4-fold difference.

To examine if DTT could reverse the effect of HS^- on DR neurons, 200 μM HS^- was applied to DR neurons held in voltage clamp near rest (V_h= -60 mV). At the peak of the HS^--induced current (an outward current in this case), DTT (527 μM) was applied. It slowly (over a period of 4 min) reversed the HS^--induced current to the level of rest and beyond, i.e induced a further inward current (Fig. 56; n=2). This suggests that DTT is capable of reversing HS^- induced effects after they have already peaked.

CHAPTER IV

DISCUSSION

I. SULFIDE EFFECTS ON THE SYNTHESIS AND STORAGE OF NEUROTRANSMITTERS.

a. Amino Acids.

Following acute poisoning with HS^-, cells are unable to utilize oxygen, resulting in hypoxia and a shift from aerobic to anaerobic metabolism. The consequence of this is an accumulation of lactate and the onset of acidosis if uncompensated (Smith and Gosselin, 1979). Under such circumstances, a large number of systems involved in neurotransmitter synthesis and metabolism will be adversely affected, particularly those dependent on mitochondrial metabolism, such as the amino acids.

To determine the alterations induced by sulfide on the metabolism of neuroactive amino acids, the total content of selected excitatory and inhibitory amino acid neurotransmitters and putative transmitter amino acids were examined in several brain regions. Significant alterations in both excitatory and inhibitory amino acid levels occurred only in the brain stem and cerebellum of rats poisoned with sulfide.

The decrease in cerebellar ASP and GLY levels following sulfide treatment may reflect an increase in tricarboxylic acid cycle activity and a more efficient channelling of the precursors to these compounds into energy production. The decrease in GLY, and probably ASP, could also be due to a decrease in the utilization of glucose as several studies have shown glucose to be the precursor molecule of GLY (Meeker and Myers, 1979; Levi et al., 1982). These decreases occurred at the lower (10 mg/kg) but not higher (30 mg/kg) doses of sulfide and may be due to the rapid nature of death following the higher dose. For instance, at higher concentrations of sulfide most animals died rapidly in contrast to the lower concentrations where most animals survived and had to be killed at a predetermined time (2 min) after exposure.

Major changes in amino acid levels occurred in the brain stem, which contains the respiratory centers. GLU, an excitatory neurotransmitter (Mayer and Westbrook, 1987; Honore, 1989; Westbrook and Jahr, 1989; Greer et al., 1990) and GABA, an inhibitory neurotransmitter (Krnjevic, 1974, Toleikis et al., 1978), both increased in the brain stem. Also, GLN, a precursor of both GLU and GABA, increased compared to control values. The increase in GLU could be due to an increased production from glycolytic and tricarboxylic acid (TCA) cycle intermediates or from the deamination of GLN. The latter mechanism is, however, unlikely as that would imply a negative coupling between GLU and GLN levels. As GABA is produced mainly from GLU by the action of glutamic acid decarboxylase (GAD), the concurrent increase in both GLU and GABA induced by sulfide suggests that the increase in GABA cannot be mainly from the decarboxylation of GLU. Another possibility is that sulfide inhibits the enzyme responsible for metabolising GABA, i.e GABA-transaminase (GABA-T) or modulates its co-factor pyridoxal phosphate. The inhibition of GABA-T activity is more likely because sulfide has been known to inhibit rather than activate a large majority of enzymes tested (reviewed by Khan, 1989). The increase in GLN could be due either to a reduced metabolism to GLU and GABA or an increased production from precursor residues of the TCA cycle (oxoglutaric acid; Cooper et al., 1991). The former mechanism is unlikely because GLU and GABA both increased instead of decreasing. A minor route of GLN production could be from amination of GLU with the utilization of ATP. This route is also unlikely to contribute much to this effect as both GLU and GABA would be expected to decrease instead of the observed increases. GLY, a putative inhibitory neurotransmitter in the brain stem and spinal cord (Champagnat et al., 1982) also increased at both doses of sulfide. This increase may represent increased synthesis from glucose as the latter has been shown to be the precursor of GLY in several brain regions (Defeudis et al., 1970; Meeker and Myers, 1979; Levi et al., 1982). Such an increased synthesis of GLY from glucose could arise from changes in glucose uptake, transport and utilization induced by sulfide. TAU, a neuro-inhibitor and potent inducer of respiratory depression (Mueller et al., 1982, Wessberg et al., 1982) was also found to increase in the brain stem following sulfide poisoning. The biochemical basis of this increase is not understood. However, this sulfide-induced increase may be of added significance in view of a recent observation, in neuroblastoma cells, where sulfide or TAU alone had no effect on sodium currents, but their combination produced a reversible abolition of the sodium currents in these cells (Warenycia et al., 1987). Thus, if sulfide's actions *in vivo* were to result in increased release of TAU, then their combined effect might produce a profound inhibition of signal transfer. ALA, a neuroactive amino acid which has been reported to increase under conditions which alter the tricarboxylic acid (TCA) cycle activity showed increases only in the brain stem. Surprisingly, the levels of ALA in the rat cerebral cortex, which are elevated following hypocapnia (Norberg, 1976), hypoxia (Norberg and Siesjo, 1975), seizures (Chapman et al., 1977) and ischemia (Folbergrova et al., 1974), all of which produce altered glucose metabolism similar to that produced by sulfide, did not change in the cortex following sulfide poisoning. This observation probably indicates the extreme susceptibility of the brain stem to the actions of sulfide.

In these studies, very little change was seen in the amino acid profiles in the cortex, hippocampus and striatum of rats after sulfide poisoning. This suggests that these three brain regions are less sensitive to sulfide in comparison to the brain stem and cerebellum. These less sensitive regions are known to have very high metabolic rates as measured by oxygen consumption, as well as a greater regional blood flow (Des Rosiers et al., 1974) than the brainstem (reviewed by Siesjo, 1978). It is thus possible that mechanisms of detoxification and rapid removal of sulfide from these regions are more efficient in disposing of sulfide (Beauchamp et al., 1984; Vetter and Bagarinao, 1989; Curtis et al., 1972). Other explanations for these observations may include selective accumulation of sulfide by different brain regions (Warenycia et al., 1988) or the differential modification of enzyme activity in these regions, as different isoforms of various enzymes are known to occur in different regions (Valentine, 1987; Vetter and Bagarinao, 1989). The selective accumulation hypothesis is supported by the fact that sulfide is much more lipophilic than hydrophillic (Windholz, 1976), and the white matter content (mainly lipid) of the brain stem and cerebellum is much higher compared to the insensitive regions which are predominantly grey matter with much less lipophilic material (Clausen, 1969). This differential lipid content suggests that sulfide will be preferentially concentrated in the cerebellum and brain stem, resulting in toxic alterations in metabolic processes in these regions. This has been confirmed by Warenycia et al. (1988) who used gas dialysis and ion chromatography to demonstrate that the brain stem accumulates twice as much sulfide compared to the other regions.

The results described in this thesis demonstrated that sulfide intoxication selectively affects brain stem excitatory and inhibitory amino acid levels, with the other regions demonstrating relative insensitivity. The selective sensitivity of brain regions to sulfide may be determined by the level of endogenous sulfide present in these regions already or the ability of different regions to handle sulfide (metabolism and clearance). Warenycia et al. (1989) have shown different regions of the brain to contain different amounts of endogenous sulfide (the brain stem having the lowest), probably a reflection of their differential abilities to metabolise and/or dispose of sulfide. Regions with high endogenous levels of sulfide may, over long periods of time, have adapted or developed protective mechanisms against the toxic actions of sulfide (Vetter and Bagarinao, 1989). For example, it has recently been suggested that, in invertebrates, one mechanism offering protection against sulfide poisoning involves a specific sulfide oxidase that limits the concentration of sulfide by converting it to sulfate (Powell an Somero, 1986; Vetter and Bagarinao, 1989). If different isozymes of this oxidase occur in different brain regions, then these regions may show different sensitivities to sulfide.

The toxicity of sulfide is frequently compared with that of cyanide. For example, sulfide has several actions that are similar to those of cyanide, such as inhibition of mitochondrial cytochrome aa_3 enzyme, and an identical LD_{50} value. Comparison of our data with those obtained following acute poisoning with cyanide (Persson et al., 1985) shows the changes in amino acid levels in various brain regions to be markedly different. For example, whereas no demonstrable changes in amino acids were seen in the cortex, hippocampus and striatum following 2 min exposure to sulfide, these regions showed marked changes in amino acids as early as 30 sec following cyanide poisoning (Persson et al., 1985). Thus, these two toxic substances may possess different temporal and neurochemical toxicity profiles, as well as selectivity towards different brain regions. It should be noted that the sulfide data do not exclude the possibility that brain sampling at times other than 2 min could show changes in amino acid levels in these regions.

These changes in amino acid levels in the brain stem may, in part, contribute to the lethality of sulfide through mechanisms involving respiratory control. Although this study demonstrated changes in the content of both excitatory and inhibitory amino acids within a time frame consistent with the known rapidity of sulfide lethality (2 min), it did not address the more important question as to whether these increases in tissue amino acid content accurately reflect changes in the release pattern of transmitters, which is important in neurotransmission and CNS function. It also could not distinguish between changes in metabolic versus transmitter pools of these amino acids. Thus, experiments utilising the local concentric push-pull perfusion technique needed to be done to delineate how changes in tissue amino acid content are translated into release from transmitter pools since the latter technique presumably recovers only transmitters that are released from transmitter pools only.

b. Biogenic Amines.

The biogenic amines, including noradrenaline, adrenaline, dopamine (catecholamines) and serotonin (5-HT, an indolealkylamine) are known to modulate respiratory rhythm generation and maintenance (Mueller et al., 1982; Murakoshi et al., 1985), motor control and memory (Pijnenburg et al., 1974; Madison and Nicoll, 1982; Bliss et al., 1983; Johnston et al., 1988), all of which show disturbances following sulfide poisoning. A dose of sulfide equivalent to twice the LD_{50} (30 mg/kg) markedly elevated noradrenaline and adrenaline levels in the hippocampus, striatum and brain stem, but not in the cerebellum and cerebral cortex. The greatest changes were in the hippocampus and may contribute to the amnesia reported in humans following sulfide poisoning (Burnett et al., 1977). This is suggested by the fact that the hippocampus has been implicated in memory processes and noradrenaline has been shown to modulate hippocampal neuronal activity (Pijnenburg et al., 1974; Madison and Nicoll, 1982; Bliss et al., 1983; Johnston et al., 1988).

In the dopamine-rich striatum, only noradrenaline and adrenaline were elevated. However, since noradrenaline has been shown to act on dopamine receptors in the mesolimbic area to produce locomotor alteration similar to that seen with dopamine (see review by Fishman et al., 1983), the increase in noradrenaline and adrenaline could contribute to motor disturbance and may account for the apparent inability of sulfide "knockdown" victims to escape from environments heavily laden with sulfide (Burnett et al., 1977).

Of greater acute toxicological relevance and consequence are the changes in the levels of brain stem biogenic amines as this region contains the respiratory centers that are responsible for the generation and maintenance of respiratory rhythms. All three catecholamines and 5-HT increased following lethal doses of sulfide. This is not surprising as the major biogenic amine synthesizing nuclei are located in the brain stem (Dahlstrom and Fuxe, 1964). Since the catecholamines and 5-HT all modulate respiratory drive (Mueller et al., 1982; Murakoshi et al., 1985), it is possible that changes of the magnitude seen in the brain stem could lead to detrimental alteration in the coupling of respiratory oscillators with consequent arrest of respiratory drive.

The mechanisms by which sulfide causes these increases are not known and were not examined in this study. However, sulfide is known to affect several metabolic enzymes (see review by Khan, 1989). The catecholamines are synthesized from the amino acid precursor tyrosine by a sequence of enzymatic steps. Tyrosine is converted to 3,4-dihydroxyphenylalanine (DOPA) by the enzyme tyrosine hydroxylase. DOPA is in turn converted to dopamine by the aromatic amino acid decarboxylase enzyme. Dopamine β-hydroxylase (DBH) then converts dopamine to noradrenaline. Depending on the presence or absence of the enzyme phenylethanolamine N-methyltransferase (PNMT), noradrenaline is then N-methylated to adrenaline or not. Tyrosine hydroxylase activation by sulfide may contribute to the increase in catecholamine levels as catecholamine biosynthesis is known to increase with changes in pH and chemoreceptor stimulation that affect this enzyme (Davis and Carlsson, 1973, Garcia DeYebenes Prous et al., 1977). Both of these effects can be produced by sulfide, via inhibition of carbonic anhydrase (Klentz and Fedde, 1978) and stimulation of peripheral chemoreceptors (Ammann, 1986) respectively. The elevations in noradrenaline and adrenaline levels could also be due to activation of DBH or PNMT respectively. DBH activation is, however, unlikely as an endogenous inhibitor of DBH (a peptide containing GLU, CYS, and GLY), which is activated by sulfide, has been reported (Duch and Kirshner, 1971). Thus, the contribution of this enzyme to the sulfide response, if any, would probably be to reduce the magnitude of the increase. Whether PNMT activity can be stimulated by sulfide is currently unknown. However, based on the fact that PNMT is the enzyme responsible for converting noradrenaline to adrenaline, one would expect changes in adrenaline to be negatively coupled to changes in noradrenaline if sulfide were activating PNMT. In this study however, all increases in adrenaline levels were accompanied by increases (and not decreases) in noradrenaline, implying that the increase in adrenaline following sulfide poisoning is unlikely to be due to activation of PNMT.

Following the synthesis or uptake (from the extracellular milieu after release) of biogenic amines, they are either repackaged into vesicles for subsequent release or are metabolised into inactive products by two enzymes, monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). MAO occurs in neurons in both particulate form in mitochondria and as a freely soluble form in the cytosol. Inhibition of either of these catabolic enzymes by sulfide can lead to rapid increases in levels of biogenic amines. Warenycia et al. (1989) have shown that sulfide inhibits MAO activity both *in vitro* and *ex vivo*. This inhibition by sulfide of MAO activity may be via interaction with sulfhydryl groups that are plentiful in this enzyme (Yasunobu and Oi, 1972), leading to persulfide formation and hence alteration in activity. This is suggested by the fact that dithiothreitol, a thiol-protecting agent (Cleland, 1964), has been shown to prevent or reverse this inhibitory action of sulfide (Warenycia et al., 1989). Although the effects of sulfide on COMT are unknown, the sensitivity of COMT to sulfhydryl reagents (Borchardt, 1977) suggests that this enzyme may be susceptible to sulfide actions.

The marked increases in levels of biogenic amines caused by sulfide, in selected brain regions, most likely through alteration in catabolic enzyme activity may contribute to the neurological sequelae of sulfide poisoning, including death after an acute toxic exposure.

II. EFFECT OF SULFIDE ON AMINO ACID RECOVERY.

At the junction between two neurones in communication (synapse), neurotransmitters can be released either spontaneously (Fatt and Katz, 1952; Katz and Miledi, 1967) or upon the arrival of an action potential at the presynaptic terminal (Kuffler et al., 1984). Both release processes depend on the entry of Ca^{2+} ions into the terminal to trigger the fusion of transmitter-filled vesicles with presynaptic terminal membrane (Katz, 1969; Augustine et al., 1987). This fusion sets up a cascade of events ultimately leading to exocytosis of the vesicular content into the synaptic gap (Augustine et al., 1987, Reichardt and Kelly, 1983). The magnitude and success of synaptic transmission depends, in part, on the quantity of transmitter released into the synaptic cleft and the duration of stay in the cleft. The duration of stay in the synaptic cleft is determined by the efficiency of uptake and metabolic processes. In earlier neurochemical studies, sulfide has been shown to alter the turnover (synthesis, storage and metabolism) of several neurotransmitters, including the biogenic amines and amino acids (Kombian et al., 1988; Warenycia et al., 1989). To determine how these changes in turnover will translate into subsequent physiological events (e.g. release and therefore synaptic transmission), the push-pull perfusion technique was used to examine transmitter release.

A large body of evidence exists to support a transmitter role for GLU and ASP as excitatory, and GABA and GLY as inhibitory, neurotransmitter amino acids in several brain regions (Krnjevic, 1974; reviewed by Ottersen and Storm-Mathisen, 1989; Mora et al., 1986). Although these amino acids are intricately involved in general metabolism (glycolysis and the TCA cycle, protein and nucleic acid metabolism), ample evidence exists to prove that transmitter and metabolic amino acid pools belong to very distinct compartments (Hamburger et al., 1981; Iadarola and Gale, 1981). The transmitter pool is readily susceptible to pharmacological manipulations such as high K^+ and electrically-evoked release and dependent on extracellular calcium ion concentration ($[Ca^{2+}]_o$), whereas the metabolic pools are less sensitive to these manipulations (reviewed by Reichardt and Kelly, 1983). Nevertheless, the interpretation of amino acid release data should be done with caution because of possible interference from the metabolic pool as it is known that metabolic sources of amino acids can be taken into transmitter pools, thus obscuring any apparent alterations in the latter (Fonnum, 1984; Ottersen and Storm-Mathisen, 1989; Storm-Mathisen and Ottersen, 1988).

In the study described in this thesis, IP injection of sulfide produced little or no change in the amount of excitatory or inhibitory amino acids recovered from all regions. Only the striatum showed an increase in the release of GABA, an abundant and important inhibitory neurotransmitter in this nucleus. This change in GABA may be responsible for, or at least contribute to, the motor disturbance observed during sulfide poisoning. The general lack of effect on amino acids could be due to the inability of this technique to detect minute but possibly important changes in transmitter levels. This insensitivity may be due to the inherent poor temporal resolution of the technique, as the dead volume in the collection tubes caused a great deal of dilution of withdrawn contents (Bayon and Drucker-Colin, 1985). It could also be that the well known rapid and efficient detoxification of sulfide removed the toxin before it had time to act (Vetter and Bagarinao, 1989; Beauchamp et al., 1984; Curtis et al., 1972). The latter reason seems to be more likely as it has been suggested earlier that for sulfide toxicity to occur, exposure levels must be high enough to overwhelm these detoxification processes (Vetter and Bagarinao, 1989; Vigil, 1979).

To circumvent the known efficient metabolic processes which may remove the systemically administered sulfide, we by-passed the systemic circulation by applying sulfide directly to the region of interest via the perfusion fluid. As various brain regions contain differing endogenous amounts of sulfide (Warenycia et al., 1989), concentrations were selected so that the amount of sulfide applied was significantly higher than the endogenous amount. Direct application of 4 μg/ml sulfide to the hippocampus caused increases in perfusate levels of both excitatory (GLU and SER) and inhibitory (GLY and ALA) amino acids. Although no changes were seen in other important amino acids (ASP and GABA), the overall changes seen following direct perfusion suggest that the lack of effect of sulfide following IP injection may be due to efficient detoxification of sulfide. It is currently not understood why only IP injection of sulfide, but not direct perfusion, caused an increase in amount of GABA recovered. One explanation could be that systemically administered sulfide interacts with both metabolic and transmitter pools whereas the selective, locally applied perfusion is affecting only the transmitter pool which is probably less sensitive to sulfide. Alternatively, the systemic route, being nonselective, could be producing a more profound GABA release in the hippocampus by interacting with other brain regions or peripheral mechanisms that influence the neuronal activity of GABA-releasing cells. Another possible, though unlikely, cause for this difference may be that sulfide in the recovered perfusate may be interfering with the separation and quantification of GABA. This does not appear to be the case as control experiments on standard amino acids, with and without added sulfide, showed no difference in the recovery of all the examined amino acids, including GABA (Kombian et al., 1988).

In the striatum, sustained increases in the amounts of recovered excitatory and inhibitory amino acids were detected following direct local perfusion with sulfide in contrast to the systemic injection where very few changes occurred. This observation lends more credence to the postulate that an efficient detoxification system in the circulation may limit the full expression of sulfide toxicity following systemic administration. Although marked changes were detected in the total amino acid content in the brain stem, the amounts of these compounds recovered *in vivo* from a selected brain stem nucleus were not affected by sulfide administration, either systemically or locally. The delayed decrease in GLY levels in this region does not appear to be toxicologically relevant as respiratory arrest and death occur about 2 min after exposure to sulfide. This lack of effect in this nucleus may reflect subregional variations (within the brain stem) in sensitivity to sulfide.

The mechanism by which sulfide may produce changes in the amount of retrieved transmitters is currently unknown and could not be addressed by this technique. Using the push-pull perfusion technique to follow changes in neurotransmitter levels in the extracellular space prior to and after sulfide treatment cannot differentiate between changes in the release process *per se* and changes in the fate of the released neurotransmitters. Following the release of amino acid transmitters, high affinity uptake systems exist that quickly transport these substances back into the nerve terminal (Davies and Johnston, 1976; Taxt and Storm-Mathisen, 1984) or into glial cells as in the case of GABA (Iversen and Neal, 1968; Reiffenstein and Neal, 1974). Inhibition of these uptake processes can lead to increases in recovered transmitters. As the GABA, and probably GLY and TAU, uptake inhibitor nipecotic acid (Hudson and Reiffenstein, 1986, Hamberger et al., 1985; Krogdsgaard-Larsen and Johnston, 1975) was always present in the ACSF, the changes in the amounts of these compounds following sulfide treatment (in the striatum and hippocampus) may reflect changes in either the release or the metabolism of released transmitters but not the uptake process. Another mechanism by which sulfide may alter amino acid recovery may be via interaction with catabolic enzymes that under normal conditions terminate the actions of these amino acids by converting them into less active products (Ottersen and Storm-Mathisen, 1989; Kugler, 1989). This is quite possible with sulfide as it has been observed to inhibit biogenic amine catabolic enzymes, leading to increased amounts of biogenic amines in selected brain regions (Warenycia et al., 1989).

Finally, sulfide could be altering the release process *per se* by interacting with presynaptic terminal release mechanisms. Since evoked neurotransmitter release requires Ca^{2+} ions and Ca^{2+} ions enter the terminals mainly through Ca^{2+} channels that are opened by changes in membrane potential (see reviews by Reichardt and Kelly, 1983; Augustine et al., 1987), sulfide could alter release by either changing the membrane potential and/or conductance (see later). Such an action of sulfide is possible because most membrane channels are rich in disulfide bridges and sulfhydryl groups which are important to their function (Fraser, 1989; Blanchard et al., 1982). Sulfide may react with these thiol groups, converting them into persulfide groups and hence changing their functional integrity (Warenycia et al., 1990; Wood, 1987).

Although the changes in neurotransmitter recovery induced by sulfide may be via any or all of the above mechanisms, these experiments could not address the question as to which of the above mechanisms discussed was responsible for or contributed to the changes detected in the amounts of these amino acids following treatment with sulfide. Furthermore, the poor temporal resolution of this technique makes it incapable of detecting transient changes in transmitter release which may be toxicologically relevant (Bayon and Drucker-Colin, 1985). Nevertheless, the study does show that sulfide may alter the extracellular levels of amino acids in the brain if adequate sulfide concentrations are achieved. It also reiterates the relative sensitivities of different brain regions to the actions of sulfide as observed in total transmitter content.

All these neurochemical studies presumably reflected only presynaptic and perisynaptic processes. However, interaction with postsynaptic processes could also produce profound alterations in neuronal, synaptic and CNS function. Thus there was the need to examine the effects of sulfide on postsynaptic neuronal processes.

III. SULFIDE ACTIONS ON SYMPATHETIC GANGLION CELLS.

Although neurochemical studies had indicated sulfide action altered several important neuronal processes, including transmitter synthesis, storage, metabolism and probably release, most of these events occurred on a time scale far too slow to adequately account for the rapid nature of HS^- lethality. Since electrophysiology is a technique that permits the observation of neuronal events on a millisecond time scale, it was employed to examine the possibility that HS^- may act to alter more rapidly occurring neuronal events that are consistent with its *in vivo* toxicity profile. The frog sympathetic ganglia preparation was chosen to test the hypothesis that HS^- would alter the electrical properties of neurons because this preparation is simple and has well characterized postsynaptic responses. HS^- was found to have an effect both on resting membrane conductances and transmitter induced responses in sympathetic ganglion cells. HS^- application led to a depolarization in sympathetic ganglion cells. Because this depolarization was insensitive to the removal of Na^+, it is unlikely that an elevation of Na^+ conductance underlies the response. However, there are several other possible mechanisms which were not examined here. 1) HS^- could cause the influx of Ca^{2+}. This possibility could be consistent with data from DR neurons in which application of the inorganic Ca^{2+} channel blocker, Cd^{2+}, blocked the depolarizing action of sulfide (see below). 2) Inhibition of a tonically active, electrogenic Na^+/K^+ ATPase by HS^- would also result in a depolarization. Evidence from the DR suggests that inhibition of Na^+/K^+ ATPase does not affect the HS^- response (see below). However, removal of extracellular K^+ which inactivates the ATPase did not affect the HS^--induced depolarizing response. In addition, it is known that the ATPase is sensitive to prolonged removal of extracellular Na^+ (Kernan, 1962), which although blocks electrogenic pump activity, had no effect on the response to HS^-. The HS^--induced depolarization could involve the suppression of a normally active K^+ conductance, such as the M-current known to be present in these cells. However, the increase in neuronal input resistance that would accompany a depolarization thus mediated would also be expected to enhance the responses to exogenously applied agonists at ion channels, such as nicotine. Because the response to nicotine was not enhanced (see below), this appears not to be the mechanism of HS^- action here. Clearly, further work is needed to elucidate the mechanism of the HS^--induced depolarization in sympathetic ganglia.

In addition to the direct depolarizing effect on frog sympathetic ganglia, HS^- also altered agonist-induced responses in these cells. Although HS^- induced a depolarizing response in these cells, it did not affect the depolarizing response induced by nicotine. Nicotinic responses at rest are due predominantly to conductance increases to Na^+ ions (the channel is nonselective for cations) via the activation of nicotinic cholinergic receptors (Weight, 1983, Hille, 1991). The nicotinic cholinergic receptor is a receptor-channel complex that is involved in rapid synaptic transmission (Hille, 1991; Kuffler et al., 1984; Marshall, 1981; Nishi and Koketsu, 1968).

The hyperpolarizing responses induced by adrenaline and muscarine were both potentiated by HS^-. In contrast to the nicotinic cholinergic response, both the adrenergic (Rafuse and Smith, 1986, Selyanko et al., 1990) and the muscarinic (Dodd and Horn, 1983; Selyanko et al., 1990) responses involve the activation of K^+ conductances. Furthermore, the receptors of both agonists (α_1-adrenoceptors and M_2 muscarinic receptors) are coupled to a K^+ channel via a G-proteins and are known to underlie slow ganglionic transmission in these cells (Nishi and Koketsu, 1968; Adams et al., 1986). A possible explanation of how HS^- potentiated the hyperpolarizing responses but not the depolarizing response could therefore be that the depolarization induced by HS^- moved the resting membrane potential further away from the potassium equilibrium potential (E_k), resulting in a greater driving force for the K^+ ions, so that subsequent activation of a K^+ conductance by adrenaline or muscarine resulted in a larger response. Another possible reason for the selective enhancement of adrenergic and muscarinic but not nicotinic response may be the fact that the nicotinic cholinergic receptor is a receptor-channel complex whereas the adrenergic and muscarine receptors (α_1 and M_2 respectively; Rafuse, 1985; Egan and North, 1986) are coupled to their channels via G-proteins, so that if sulfide acted primarily on G-proteins, a selective action would result. Sulfide thus appears to selectively enhance only K^+ conductances.

The lack of effect on the nicotine-induced response (a predominantly Na^+ conductance) is not unique as earlier workers observed that HS^- did not effect the TTX-sensitive Na^+ channels in neuroblastoma cells (Warenycia et al., 1989). Interestingly, the TTX-sensitive Na^+ channels were blocked when HS^- was applied in the presence of the amino acids taurine and cysteine. It would be of interest to determine whether this combination also affected the nicotinic response. This was however, not tested. In the hippocampus, HS^- does not also alter Na^+-dependent active neuronal properties such as the action potential amplitude and duration in CA1 cells (Baldelli, 1990). Since the adrenergic and muscarinic responses are responsible for slow ganglionic transmission, while the nicotinic response is generally involved in fast synaptic transmission in these cells (Marshall, 1981; Blackman et al., 1963; Nishi and Koketsu, 1968; Adams et al., 1986), HS^- appears to selectively alter only slow synaptic events in these cells in contrast to hippocampal CA1 cells where it affects fast synaptic responses (EPSP) mediated by amino acids.

In addition to potentiating the adrenaline response and the muscarine-induced slow synaptic responses in sympathetic ganglion cells, HS^- also produced a delayed potentiation of the K^+-activated pump response. The delayed effect of HS^- on the Na^+/K^+ ATPase pump response suggests that HS^- inhibits an aspect of the pump, which may contribute to the initial depolarization, and which upon relief of the inhibition, reactivates with a significant contribution to the membrane potential. This could be an indirect effect through the activation or inhibition of regulatory proteins or the direct inhibition of enzymes involved in the process. It does not appear to be an effect on metabolic enzymes (ATP generating enzymes), as cells subjected to repeated and extended periods of exposure to sulfide do not require ATP supplementation to produce this effect. Also, most of the slow synaptic responses (adrenergic and muscarinic included) are known to be very sensitive to metabolic inhibitors (Kobayashi and Libet, 1968). If HS^- were acting to inhibit metabolic enzymes, then these responses should be attenuated and not potentiated. These observations suggest that HS^- may be inhibiting only the enzyme involved in the utilization of ATP to drive the pump i.e Na^+/K^+ ATPase. This is probably the case as HS^- has been shown to inhibit this enzyme *in vitro* (Khan, 1989). Furthermore, in DR and hippocampal CA1 cells, the inhibition of the Na^+/K^+ ATPase with strophanthidin, abolishes the HS^--induced pump-dependent washout hyperpolarization and its underlying current.

This study demonstrated that HS^- had direct effects on sympathetic ganglion cell conductances and modulated slow neurotransmitter-mediated synaptic responses but not fast ones. The effect on the Na^+/K^+ ATPase pump activity also showed that HS^- may interact with other neuronal electrochemical processes. The mechanisms by which HS^- produced all of these effects in sympathetic ganglia are not known and could not be thoroughly examined by this technique (extracellular recording). However, if these peripheral effects of HS^- were to occur in CNS nuclei *in vivo*, then HS^- will first excite neurons (depolarization), which in the presynaptic terminal will lead to increased transmitter release and then subsequently, produce a prolonged state of hyperpolarization (potentiation of transmitter-induced hyperpolarizations) leading to suppression of synaptic transmission and cessation of central regulatory processes. This hypothesis is consistent with *in vivo* toxicity actions of sulfide as increased respiratory rate precedes respiratory arrest in HS^- poisoned victims (Ammann, 1986).

The increase in respiration could also be due to the action of sulfide on peripheral chemoreceptors (Haggard et al., 1922; Smith, 1989).

IV. *IN VITRO* ELECTROPHYSIOLOGICAL ACTIONS OF SULFIDE ON DORSAL RAPHE NEURONS.

Toxicologically relevant concentrations of HS^- produced four different effects on dorsal raphe (DR) neurons *in vitro*. These effects of sulfide on the postsynaptic membrane conductances are concentration-dependent, with concentrations of 30 μM and below producing no measurable change in resting conductance while 300 μM produced a maximal effect on membrane conductances. A large percentage of DR cells (43%) responded to HS^- with a rapid onset, weakly voltage-dependent inward current (depolarization) with a slight change in steady-state conductance. Approximately 14% responded to HS^- with a slow onset, voltage-dependent outward current (hyperpolarization) accompanied by an increase in conductance. A third group of cells (~30%) responded to HS^- with a biphasic response, first a rapid onset inward current which was then rapidly superceded by a sustained outward current. All these effects were reversible upon removal of HS^- and were usually (> 90%), but not always, followed by a washout outward current (hyperpolarization). The final group of DR cells (13%) neither had any observable postsynaptic response to HS^- nor was its removal ever followed by a washout outward current.

Irrespective of the postsynaptic response of a DR cell to HS^-, the latter always produced a dose-dependent inhibition of the complex synaptic potentials and their underlying currents evoked in these cells. In DR cells without membrane responses to HS^-, measurable though less profound inhibition of synaptic responses (~20% reduction in IPSP in such cells compared to the > 80% inhibition in responding cells at 200 μM HS^-) were observed. This would suggest that in addition to the readily measurable postsynaptic actions, HS^- also has presynaptic effects on nerve terminals or axons. HS^- may therefore, through both pre- and postsynaptic mechanisms, alter or impair neuronal function leading to toxicity. The fact that sulfide has also been shown to have similar actions on the CA1 pyramidal neurons (Baldelli, 1990), which have greatly different membrane properties than the DR neurons, is evidence that this agent can elicit similar effects in very different nerve cells. It is thus conceivable that HS^- could induce similar effects in respiratory related neurons as observed in DR and CA1 pyramidal cells; these effects would lead to the uncoupling of these neurons and thus to a cessation of respiratory drive and death (Evans, 1967; Ammann, 1986).

a. Mechanism(s) of the Sulfide-induced Outward Current.

In 14% of DR cells studied, sulfide induced a slow onset (60-90 sec), voltage-dependent outward current with an increase in conductance recorded over the entire voltage range examined (-115 to -40 mV). The reversal potential for this conductance increase, determined as the point of zero relative to current, was -86.7 ± 3.0 mV (mean ± SEM) in normal (2.5 mM K^+) saline. This point is the equilibrium or reversal potential (E_{rev}) and shifted in the positive direction with changes in extracellular K^+ ion concentration ($[K^+]_o$). At the more positive potentials (-50 to -40 mV), HS^- also either inhibited an outwardly rectifying K^+ conductance or activated a net inward current. The sulfide-induced outward current was partially blocked by extracellular Ba^{2+} while a combination of Ba^{2+} and Cs^+ ions (extracellular) completely eliminated the outward current and the accompanying conductance increase. This combination also ummasked an inward current induced by sulfide. Furthermore, cadmium (Cd^{2+}), an inorganic calcium channel blocker, eliminated the current and the accompanying conductance increase with the unmasking of an inward current.

Finally, the sulfide-induced outward current was sensitive to the removal of extracellular Ca^{2+} ions.

In excitable cells, the activation of outward current, accompanied by an increase in conductance is either due to increased K^+ efflux or Cl^- influx, both of which normally have reversal potentials negative to the resting membrane potential. Outward currents oppose excitation, leading to stabilization of membrane potential (review by Rudy, 1988, Hille, 1991). The Cl^- reversal potential is usually close to resting membrane potential (± 15 mV; Hille, 1991). As DR cells rest between -55 and -70 mV, if the HS^- response was due to activation of a Cl^- channel, then the reversal potential would be between -40 to -85 mV. However, under the recording conditions of these experiments (KCl-filled electrodes), Cl^- diffused into the cells, thus shifting the Cl^- reversal potential to a more positive potential (Williams et al., 1988). Also in whole-cell patch recordings using a K^+-gluconate containing intracellular solution with no Cl^-, the E_{rev} of the sulfide-induced conductance increase was identical to that observed in experiments using KCl-filled electrodes (Figs. 32, 35 36). It is therefore unlikely that the outward current was carried by Cl^- ions. Thus, the data suggests that the observed outward current activated by sulfide is due to an increased conductance to K^+.

In DR cells, the estimated E_K is about -105 mV (Williams et al., 1988). The HS^--induced K^+ conductance has an E_{rev} more positive than that reported for these cells, which may suggest either an underestimation of the E_{rev} by this technique or the activation of more than one conductance by HS^-. The former possibility is unlikely as precautions were taken to optimize the clamp and also, the previous E_K (Williams et al., 1988) determinations were made using this same technique. The latter possibility is more likely to be the case, namely that sulfide causes the concomitant activation of an inward conductance. This is supported by the observation here that sulfide always elicits an inward current in cells in which the outward K^+ currents have been blocked (Figs. 34, 36B and 43). This masked inward current may therefore be responsible for the observed difference towards the positive in the E_{rev} of the HS^--induced outward current from the estimated E_K.

The shift in the positive direction of the E_{rev} of the sulfide-induced outward current with increasing $[K^+]_o$ is not according to that predicted from the Nernst equation (a 61 mV shift per decade change in $[K^+]_o$ at 34 °C; Hille, 1991). The dependence of the E_{rev} of the sulfide-induced outward current on $[K^+]_o$ however, supports the hypothesis that the conductance increase is due in part to the activation of K^+ channels. The lack of concordance with the theoretical shifts could be due to the concurrently activated inward current that appears to be induced by HS^- in all responding DR cells. As the nature of the inward current has not been well characterized, it is difficult to predict its overall effect on the outward current.

i. What Type of K^+ Channel(s) are Activated by Sulfide?

At least fifteen types of K^+ channels have been described and characterized since the delayed outwardly rectifying K^+ current was first described by Hogkin and Huxley (1952) in squid giant axon. These channels show a great deal of diversity and ubiquity, displaying varying voltage and agonist sensitivities, pharmacological properties and physiological roles (see review by Rudy, 1988). Certain monovalent and divalent cations, organic compounds and toxins have been found to selectively block some of these channels (review by Rudy, 1988; Hille, 1991) when applied either extra- or intracellularly. These selective blockers were used in this study to determine the type(s) of K^+ channels activated by sulfide in the DR nucleus.

ii. Calcium-Activated K^+ *Conductance* (gK_{Ca}).

Extracellular application of Ba^{2+} produced only a partial block of the HS^--induced outward current without much change in the HS^--induced conductance increase on DR cells. However, a combination of Ba^{2+} and Cs^+ completely blocked both the HS^--induced outward current and the conductance increase in DR cells. The effects of Cs^+ alone were not tested here. In addition to this block, the combination unmasked an inward current in these cells. The inhibition of the sulfide-induced outward current by Ba^{2+} and Cs^+ is further evidence that the outward current is due to activation of a K^+ conductance by sulfide. The partial block of this current by extracellular Ba^{2+} alone may be due either to insufficient intracellular levels of Ba^{2+} as it is more effective from inside (Hille, 1991) or the presence of Ba^{2+}-insensitive K^+ current components. As both Ba^{2+} and Cs^+ are known to block several K^+ channels (Bezanilla and Amstrong, 1972; Hille, 1973; Colmers et al., 1982, Johnston et al., 1980, Puil and Werman, 1982), especially the inwardly rectifying K^+ channels (Williams et al., 1988), these results did not implicate any particular type of K^+

channel in the sulfide response. Because calcium-activated potassium conductances are blocked by both Ba^{2+} and Cs^{+} (reviewed by Rudy, 1988; Hille, 1991), we examined if this conductance was activated by sulfide to produce or contribute to the outward current by employing more specific tests.

As gK_{Ca} has been shown to be activated by Ca^{2+} ions and sensitive to removal of extracellular Ca^{2+} (Barrett and Barrett, 1976; Junge, 1980; Storm, 1986) we deleted Ca^{2+} from the extracellular perfusate and examined the actions of sulfide. The deleted Ca^{2+} was replaced by an ACSF containing a high concentration of Mg^{2+}. The sulfide-induced outward current was completely eliminated in the presence of the ACSF containing a nominal Ca^{2+} and high Mg^{2+} ion concentration. The above observation implicated extracellular Ca^{2+} in the sulfide-induced outward current and suggested that a calcium-activated potassium current (I_{KCa}) was activated by sulfide. Because extracellular calcium is known to play a role in membrane stabilization (Hille, 1991; Kuffler et al., 1984), it was important to show that the elimination of the outward current in the absence of calcium was not due to membrane destabilization. Since extracellular calcium enters the cell mainly via calcium channels, an inorganic calcium channel blocker, cadmium (Hagiwara and Takahashi, 1967; Yarom et al., 1985; Hille, 1991) was used to block calcium channels and the effect of sulfide examined. In the presence of normal extracellular calcium (2.4 mM), Cd^{2+} completely eliminated the sulfide-induced outward current, and unmasked a typically small inward current. This observation proved that the results obtained in nominally zero Ca^{2+} solution were not due to nonspecific membrane destabilizing effects. It further implied the involvement of extracellular calcium in the sulfide-induced outward current response while suggesting that sulfide may induce calcium influx into these cells. The above observations strongly implicate the calcium-activated potassium channel in the sulfide-induced outward current response.

Calcium-activated potassium conductance (gK_{Ca}) has been described in several tissues and cells (Meech and Strumwasser, 1970; Barrett and Barrett, 1976; Meech, 1976; Hille, 1991) and shown to be induced by internal Ca^{2+} ions either from influx from the extracellular milieu (review by Rudy, 1988; Hille, 1991) or from internal release from intracellular storage sites (Trautman and Marty, 1984; Bubinsky and Oxford, 1985; Krnjevic and Xu, 1989). Although not carefully examined, the relative contribution of intracellular calcium sources to this response is probably very minimal as both removal of extracellular calcium and application of an inorganic calcium channel blocker (Cd^{2+}) eliminated the sulfide-induced outward current.

Several types of gK_{Ca} have been described but only two are well characterized, having greatly different kinetics, Ca^{2+} sensitivities, pharmacology and physiological roles (see review by Rudy, 1988; Hille, 1991). The large conductance, less Ca^{2+} sensitive channel has been called maxi-K, while the small conductance, highly Ca^{2+} sensitive channel is frequently referred to as SK or I_{AHP} because its activation underlies the slow afterhyperpolarization following the firing of an action potential (Yarom et al., 1985; Lancaster and Nicoll, 1987; Hille, 1991). Both these channels can coexist in the same cell (Pennefather et al., 1985; Romey and Lazdunski, 1984) and may both contribute to the sulfide-induced response in the DR nucleus. Although their relative contributions to the sulfide-induced current was not examined in this study, they can be pharmacologically isolated and studied as they show different sensitivity to both organic and inorganic blockers (Kolb, 1990; Dreyer, 1990; Hille, 1991).

iii. Voltage-Activated K^+ Channels.

The sensitivity of the sulfide-induced outward current to the blocking actions of Ba^{2+} and Cs^+ would suggest the involvement of voltage-activated K^+ channels as these cations are known to block most K^+ channels (reviewed by Rudy, 1988; Hille, 1991). Several voltage-activated K^+ channels have been described in both vertebrate and invertebrate neurons (see review by Rudy, 1988, Hille, 1991). The prototypes of this class of channels are the delayed rectifiers (I_K) and the fast, transient, 4-aminopyridine (4-AP)-sensitive channel (I_A). Although not examined in this study, the I_A is not likely to contribute to the outward current induced by sulfide on resting DR cells. This is suggested by earlier observations in CA1 pyramidal cells where sulfide induces a similar outward current; 4-AP at a concentration sufficient to entirely block I_A, did not affect the sulfide-induced outward current (Baldelli, 1990). Furthermore, because I_A rapidly activates (within 3-5 msec) and inactivates (<100 msec at room temperature; Rudy, 1988; Segal and Barker, 1984), the voltage ramp protocol employed in this study is too slow to measure any substantial contribution from I_A as it will activate and inactivate before the steady-state current is achieved.

Baldelli (1990) also showed that extracellular tetraethylammonium (TEA; 50

mM; twice the concentration known to block I_K in these cells; Segal and Barker, 1984) did not affect the HS^--induced outward current. Furthermore, sulfide did not affect action potential characteristics of CA1 pyramidal cells that are determined in part, by I_K. Also, in DR cells, sulfide induced a net inward current in the voltage range between -50 and -40 mV. Since this range approximates the region where most outwardly rectifying K^+ conductances, especially the I_K channels are likely to activate, sulfide may be acting to inhibit such currents rather than activate them. Alternatively, activation of inward currents in this voltage range may account for the net inward current response to sulfide. Thus, although both I_K and I_A are sensitive to blockade by Cs^+ (Rudy, 1988), which blocked the HS^--induced outward current, these channels are not likely to contribute to the HS^- response.

iv. Inwardly Rectifying K^+ Channels.

The sensitivity of the sulfide-induced outward current to the blocking actions of Ba^{2+} and Cs^+ ions also strongly suggested a contribution of inwardly rectifying potassium conductances to this response. In the DR, three inward rectifiers have been described by Williams et al., (1988). There is the slow, hyperpolarization-activated, non-inactivating, K^+- and Na^+-dependent I_Q or I_h which has been described in other excitable cells including neurons and muscles (Mayer and Westbrook, 1983; Crepel and Penit-Sorea, 1986; Benham et al., 1987; McCormick and Pape, 1990a & b). This conductance is carried by both Na^+ and K^+ ions and is sensitive to blockade by Cs^+. The reversal potential for I_Q is about -40 mV in thalamic relay neurons

(McCormick and Pape, 1990). Based on the kinetics of this channel, and the fact that the HS^--induced outward current was sensitive to Cs^+, the activation of this channel may contribute to the sulfide response. However, because the activation of I_Q results in a net inward current (McCormick and Pape, 1990) at the resting membrane potential, its activation by sulfide would serve to antagonize the outward current produced by sulfide.

Another voltage-dependent inwardly rectifying K^+ conductance (I_{IR}) in the DR is similar to others described in several other tissues (Constanti and Galvin, 1983; Hille, 1991). This rapidly activating channel rectifies around E_K in contrast to I_Q, which rectifies at more positive potentials, presumably because it is carried by two ions. I_{IR} is also sensitive to blockade by both Ba^{2+} and Cs^+ (Williams et al., 1988). The HS^--induced outward current was sensitive to both Ba^{2+} and Cs^+ suggesting that activation of I_{IR} may contribute to this effect. This is unlikely because of the shape of the I-V curves which do not show any inward rectification.

Finally HS^- could activate, directly or indirectly through release of 5-HT, a 5-HT_{1A}-mediated conductance. This conductance has been shown by Williams et al., (1988) to be activated by both 5-HT and GABA (via 5-HT_{1A} and $GABA_B$ receptors respectively). It was further shown to be distinct from the other inward rectifiers in the DR, especially in its voltage-dependence and sensitivity to pertussis toxin. HS^- may activate this conductance as part of the outward current. This is suggested by the similarity between the HS^- and 5-CT induced outward currents. For example, the 5-CT induced conductance increase is only partially sensitive to blockade by Ba^{2+}

(Williams et al., 1988) as is the case with the HS^- induced conductance increase. Furthermore, the application of sulfide in the presence of a submaximal concentration of 5-CT (100 nM) did not result in any additional outward current suggesting that these two compounds may be acting on the same K^+ channel. However, the sulfide-induced current does not demonstrate an inward rectification in the voltage range that $I_{5\text{-}CT}$ usually rectifies. In addition, isolation (by subtraction) of the Ba^{2+} and Cs^+ sensitive component of the HS^--induced current also suggests that the lack of inward rectification was not due to properties of the concomitantly activated inward current (Figs. 34, 42). Sulfide and 5-CT may thus be interacting in an unknown way to produce the observed effect. The indirect activation of $I_{5\text{-}CT}$ through the presynaptic release of 5-HT by HS^- is unlikely as cyanopindolol, a 5-HT_1 receptor antagonist (Bobker and Williams, 1990) did not affect the sulfide-induced outward current (not shown). Thus, although sulfide may act on DR cells to activate different inward rectifiers to contribute to the outward current, particularly the I_{IR} and $I_{5\text{-}CT}$, this contribution to the overall response is probably minimal as the net sulfide-induced outward current showed outward rectification with little inward rectification at the more negative potentials (Fig. 33).

v. Other K^+ Channels.

In addition to the above channels discussed, other K^+ channels have been described that could potentially contribute to the HS^--induced outward current. Activation of a leak conductance, which is probably carried largely by K^+ ions, such as described in bullfrog sympathetic ganglia (Adams et al., 1982; Jones, 1989), could contribute to the HS^--induced outward current. Jones (1989) demonstrated that the resting potential of frog sympathetic ganglia depended mainly on this current which is relatively voltage-insensitive, not blocked by most K^+ channel blockers, but depends on extracellular K^+ ion concentration. Because the HS^--induced outward current does demonstrate voltage-dependence and is sensitive to Ba^{2+} and Cs^+, it shares few known properties of a leak conductance. It is therefore unlikely that a leak conductance contributes significantly to this response in DR cells.

Although the presence in DR cells of the voltage-activated K^+ current that is inhibited by muscarine (I_M) is unknown, activation of such a current could be responsible for the HS^- evoked outward current. Like the sulfide response, I_M is blocked partly by Ba^{2+} (Halliwell and Adams, 1982; Hille, 1991). In addition, it has been shown that activation of I_M can underlie the hyperpolarization mediated by somatostatin in hippocampal neurons (Moore et al., 1988; Watson and Pittman, 1988). Furthermore, the activation of a muscarine-sensitive conductance in CA1 pyramidal cells leads to a K^+ current which rectifies inwardly and is insensitive to Cs^+ (Pittman and Siggins, 1981) which partially blocks the HS^--induced outward current in CA1 pyramidal cells. Furthermore, the outward current induced by HS^- in CA1 cells is insensitive to carbachol, an acetylcholine analogue that activates muscarinic receptors (Baldelli, 1990). As the outward current induced by HS^- in pyramidal cells and DR cells is quite similar, it appears unlikely that a muscarine-sensitive current is involved in this response to HS^-.

The well known ability of HS^- to inhibit mitochondrial oxidative phosphorylation (Beauchamp et al., 1984; Finklea, 1977, Smith, 1979), could lead to depletion of intracellular ATP and activation of the recently described ATP-sensitive potassium conductance (gK_{ATP}). This channel, which is voltage-insensitive and is permeated mainly by K^+, has been described in several excitable tissues including cardiac muscle (Noma, 1983; Kakei et al., 1985), skeletal muscle (Spruce, 1987) and neurons (Ashford et al., 1988), as well as in pancreatic beta cells (Cook and Halles, 1984; Ashcroft et al., 1984; Rorsman and Trube, 1985). gK_{ATP} is inhibited by adenosine-5'-triphosphate (ATP) and sulfonylurea compounds such as tolbutamide and glyburide and activated following decreased ATP availability (Hille, 1991). The possibility that sulfide activated a gK_{ATP} in DR cells via depletion of ATP is unlikely as we could still record (for extended periods) the sulfide-induced outward current in cells held in the whole-cell recording mode, with the patch electrode filled with an intracellular recording solution containing 5 mM ATP which freely diffuses into the cell.

Another K^+ current that has only recently been discovered in both vertebrate and invertebrate neurons is a Na^+-activated K^+ conductance (gK_{Na}; Martin and Dryer, 1989; Haimann et al., 1990). This current has also been recorded in mammalian cortical cells (Schwindt et al., 1989) and brainstem neurons (Dryer et al., 1989). The channels that pass the K^+ current are gated by intracellular Na^+ ions and are partly activated at physiological intracellular Na^+ concentration (4-12 mM) and may therefore contribute to RMP (Haimann et al., 1990; Dryer et al., 1989). The action of sulfide to induce an inward current in all DR cells and an outward current in some could be consistent with the activation of a Na^+ current that activates a gK_{Na} in some DR cells. Since 4-AP and TEA, which block this current (Dryer et al., 1989; Bader et al., 1985) do not block the sulfide-induced outward current in CA1 cells (Baldelli,1990) and since the latter effect is similar to that in DR cells, it is unlikely that this current contributes to the sulfide-induced outward current in DR cells.

In general therefore, the HS^- activated outward current (hyperpolarization in current clamp) is very similar to those induced in hippocampal CA1 cells by HS^- (Baldelli, 1990) and by anoxia (Hansen et al., 1982; Fujiwara et al., 1987; Leblond and Krnjevic, 1989). The type of K^+ channel involved in the anoxia-induced outward current is presently unknown. Fujiwara et al., (1987) proposed the activation of a voltage-independent conductance such as the gK_{ATP}, based only on indirect evidence. Leblond and Krnjevic (1989) did not observe any change in hyperpolarization in the presence of high intracellular concentrations of ATP (100-200 mM). Krnjevic and Xu, (1989) provided evidence to suggest that this anoxic response in CA1 hippocampal cells is due to the activation of a calcium sensitive potassium conductance i.e gK_{Ca}. They proposed, based on the suppression of the anoxic response by extracellular dantrolene sodium, which blocks the release of Ca^{2+} from intracellular stores (Desmedt and Hainaut, 1977) that the source of calcium was from the internal stores. Our evidence suggests an extracellular source for the calcium mediating the HS^--induced outward current. If this HS^- response is solely due to extracellular Ca^{2+}, this will be an interesting and important observation as hydrogen cyanide, a toxic substance which has long been thought to share a common mechanism of toxicity with sulfide (Smith, 1977, Beauchamp et al., 1984), has also been shown to activate a gK_{Ca} but through the release of calcium from intracellular sites in carotid type I chemosensory cells (Biscoe et al., 1988; Biscoe and Duchen, 1989; Biscoe et al., 1989). The source of Ca^{2+} for the activation of gK_{Ca} would be another difference between HS^- and cyanide (see earlier discussion) in their toxicity profiles. This difference may however only reflect differences in calcium homeostasis between peripheral and central neurons.

b. Mechanism(s) of the Sulfide-induced Inward Current.

In 43% of DR cells studied, HS^--induced a rapid onset inward current (depolarization) which was weakly voltage-dependent, and with very little or no change in conductance. This inward current was always followed by a washout outward current (hyperpolarization) after the removal of sulfide. It was neither blocked nor potentiated by Ba^{2+} (not shown but see fig. 43). Preliminary tests with Cd^{2+}, an inorganic calcium channel blocker, showed it to partly block this current but did not alter the I-V relationship (not shown). Furthermore, in DR cells which respond to sulfide with an outward current with no apparent inward current, any manipulation (such as application of Ba^{2+}, Cs^+ and Cd^{2+}) that eliminates the outward current usually unmasked an underlying sulfide-induced inward current. However, the deletion of Ca^{2+} from the extracellular medium which eliminated the sulfide-induced outward current did not unmask an inward current. These observations are consistent with the hypothesis that the sulfide-induced inward current may be carried, at least in part, by calcium ions.

The weak voltage-dependence of the current and the ensuing washout outward current following the removal of sulfide also suggested it might be due to the inhibition of the electrogenic Na^+/K^+ ATPase. Because activation of this pump leads to a net outward current, its inhibition produces a relative inward current (depolarization). Conversely, disinhibition of the pump will cause an outward current (hyperpolarization) due to pump reactivation (Rang and Ritchie, 1968; Smith et al., 1986; reviewed by Phillis and Wu, 1981). We therefore tested the hypothesis that the inward current was due to the inhibition of the Na^+/K^+ ATPase by examining the response to HS^- in neurons whose Na^+/K^+ ATPase had been blocked with strophanthidin (Yoda and Hokin, 1970). Strophanthidin (7 μM) completely eliminated the washout outward current that normally follows the removal of sulfide. However, the inward current induced by sulfide was not affected. Therefore the inward current induced by sulfide is independent of the activity of the Na^+/K^+ ATPase.

Based on the above, the absence of a marked change in conductance accompanying the inward current is puzzling. One possible explanation may be that more than one conductance is affected. Inward currents can be produced by the activation of Ca^{2+} and/or Na^+ channels or by the suppression of a tonically active outward current. The activation of channels such as those which carry the I_Q or I_H can also produce an inward current (Yanagihara and Irisawa, 1980; Mayer and Westbrook, 1983; McCormick and Pape, 1990). The nature of this current is not clear from the present experiments. Because of its weak voltage-dependence, and the limitations of the voltage clamp we could attain, it was not possible to reliably estimate the reversal potential of the current.

c. On the Mechanism(s) of the Biphasic Response.

30% of DR cells responded to HS^- with an initial rapid onset inward current (depolarization) which was then superceded by a sustained outward current (hyperpolarization). The characteristics of the inward current were identical to those observed in the monophasic inward current cells. The initial inward current had a rapid onset and apparently remained tonically activated as it could be unmasked by inclusion of Ba^{2+} and Cs^+ in the perfusate. The I-V relationship of this isolated current revealed an inward shift and a similar weak voltage-dependence with little or no change in slope conductance as was observed in cells responding to sulfide with an inward current only. This early phase was independent of Na^+/K^+ ATPase activity as strophanthidin, although eliminating the washout outward current, did not block it. The similarity between this inward current and that recorded in DR cells responding to sulfide with an inward current suggest they share a common underlying mechansim.

The later outward current phase in cells responding biphasically to sulfide activated about 60-90 sec after the onset of the early inward current, rapidly attained a peak level and remained at this level as long as sulfide was present. The latency to onset of the outward current in biphasic responders was the same as for cells responding with only an outward current. The steady-state I-V relationship measured at the peak of this sulfide-induced current revealed a voltage- dependent current accompanied by an increase in conductance. This current reversed at about -78 mV, depended on extracellular $[K^+]_o$ and was completely blocked by a combination of Ba^{2+} and Cs^+. These attributes of the late outward current phase suggested it was due to the activation of a potassium conductance. The positive shift in the E_{rev} may be due to the concurrent activation of other cation conductances (the early phase). Thus, the onset of activation, voltage dependence and antagonist sensitivity of this current are similar to that recorded in cells responding to sulfide with an apparent outward current only. This suggests that the inward current is present in essentially all (87%) DR neurons although it may be proportionally greater in biphasic cells than in those responding to HS^- with a monophasic outward current. The outward current response appears to only be expressed in some neurons responding to HS^-, and like the inward current, appears to be expressed in different proportions in different cells.

The blockade of the outward current in cells responding to sulfide with either biphasic or monophasic outward current always unmasked an inward current with similar properties. As the neurons selected for this study exhibited very similar physiological properties, it suggests that sulfide treatment can reveal a hitherto unsuspected heterogeneity in the physiological properties of DR neurons.

d. Sulfide Effects on Non-Responding Dorsal Raphe Cells.

In 13% of all DR cells studied, application of sulfide did not produce a measurable postsynaptic membrane effect at rest, even at high concentrations (300 μM). There were also no voltage-dependent effects in these cells, as the steady-state I-V relationship measured in control and in the presence of sulfide were superimposable. Based on the observation that some cells responded to HS^- with both an inward and an outward current, it was hypothesized that these cells may belong to a group with a perfectly balanced outward and inward current components (in terms of degree of sensitivity, latency of activation and magnitude of response). To test this hypothesis, we attempted to block a putative outward current component with Ba^{2+} and Cs^+. However, under these conditions, no inward current was observed suggesting the absence of a response to HS^- was absolute. Interestingly, the synaptic responses recorded in these cells were sensitive to the actions of sulfide. This synaptic effect may be due to a presynaptic action of sulfide that results in decreased transmitter release.

As these cells also satisfied all the criteria that all DR cells were subjected to prior to experimentation (see Methods), it appears likely that they represent DR serotonergic cells. In the context of the monophasic and biphasic cells, it is suggested that the non-responders represent a subpopulation of DR serotonergic cells that lack the mechanisms or the sulfide sensitivity that the other DR neurons express to varying degrees.

d. On the Mechanism(s) of the Washout Outward Current (Hyperpolarization).

Most DR cells that responded to HS^- produced a washout outward current (hyperpolarization) 1-3 min after the removal of HS^-. The magnitude of this current differed from cell to cell and depended also on the type of response induced by sulfide. For example, monophasic inward current cells always had robust washout outward currents while monophasic outward cells had the smallest washout currents (Figs. 29 and 39). Biphasic cells had washout currents of intermediate amplitude although they increased to magnitudes comparable with those of the monophasic inward cells in the presence of Ba^{2+} and Cs^+ (Figs. 42 & 43) suggesting the amount of washout current depended on the net inward current. This washout outward current was slightly sensitive to changes in extracellular K^+ ion concentration but was largely voltage independent. These observations suggest the involvement of an electrogenic pump current, the commonest in neurons being the Na^+/K^+ ATPase pump that is driven by ATP (Phillis and Wu, 1981). This hypothesis was confirmed by the ability of strophanthidin, a specific Na^+/K^+ ATPase inhibitor (Yoda and Hokin, 1970), to abolish the washout outward current. This observation implied that HS^- inhibited this pump, producing an inward current as a consequence. The disinhibition and reactivation of the pump after the removal of HS^- then produced the washout outward current (hyperpolarization). This hypothesis is supported by the fact that the pump is known to be readily inactivated, for example by removal of extracellular K^+ ions, and reactivates rapidly upon the re-introduction of K^+ ions (see review by Phillis and Wu, 1981; Rang and Ritchie, 1969; Smith et al., 1986). The actions of HS^- on this pump appear to be through an interference with the pump mechanism such as inhibition of the ATPase rather than via the depletion of ATP supply. This hypothesis is supported by the observation that sulfide has been shown to inhibit the ATPase *in vitro* (see review by Khan, 1989). Furthermore, intracellular application of high concentrations of ATP did not prevent the sulfide-induced washout response. This hypothesis is contrary to the long held views that acute HS^- toxicity results from energy deprivation arising from its inhibition of oxidative phosphorylation (Finklea, 1977; Smith, 1979).

The occurrence of pump-dependent washout outward current appears to be a universal phenomenon in nerve cells in response to substances or manipulations that induce a state of altered oxygen utilization. For example, a similar strophanthidin-sensitive washout hyperpolarization (outward current) was observed in CA1 hippocampal cells following removal of HS^- (Baldelli, 1990) and during reperfusion with oxygenated medium after inducing anoxia or hypoxia (Fujiwara et al., 1987; Leblond and Krnjevic, 1989). A similar phenomenon was also observed in frog sympathetic ganglia after treatment with high concentrations of sulfide. The overall consequence of pump inhibition by HS^- will be an increase in net inward current which could lead to alterations in cell function.

e. Effect of Sulfide on Synaptic Transmission.

Another mechanism by which sulfide could alter CNS function is by inhibition of synaptic transmission. To determine if sulfide inhibited synaptic responses in the DR nucleus, different concentrations of sulfide were applied and the synaptic potentials or their underlying currents recorded. Concentration-response curves were then constructed for each of the three components of the complex synaptic responses elicited by focal electrical stimulation near the DR. Data for these curves were pooled from all three different types of cells with postsynaptic responses to sulfide i.e without regard for the type of postsynaptic response induced by sulfide.

The results in this study indicate that HS^- reversibly inhibits all three components of the complex synaptic response evoked in DR neurons in a dose-dependent manner. All three components were insensitive to concentrations of sulfide below 30 μM while 300 μM produced a maximal inhibition of all synaptic responses. The different components of the synaptic response were inhibited with different potencies. For example, the sEPSC was more potently inhibited with an estimated EC_{50} value of 45 μM while the IPSC was least potently inhibited with an EC_{50} of 103 μM. The DSC was inhibited with an EC_{50} value of 83 μM. The dose-dependence of this effect suggests HS^- interacts with specific elements in the DR in a way that obeys the laws of mass action. Furthermore, the fact that sulfide inhibits the synaptic responses in DR neurons with different potencies suggests that the several components of the complex response exhibit differential sensitivity to the actions of HS^-.

The inhibition of synaptic responses by sulfide could be via either presynaptic or postsynaptic mechanisms or a combination of both. Sulfide could be acting presynaptically to alter the release and hence, availability of neurotransmitters to act on their postsynaptic receptors. The presynaptic actions of sulfide on synaptic responses is suggested by the observation that a very low concentration of HS^- (30

μM; Fig. 40) which does not produce a measurable change in postsynaptic conductance, induced a measurable decrease in synaptic response. Furthermore, in DR cells with no detectable postsynaptic response to HS$^-$, decreases in synaptic currents of magnitudes comparable to those recorded in responding cells could be recorded at a sulfide concentration of 30 μM. The presynaptic action of HS$^-$ could not be directly examined due to the technical difficulty of recording from presynaptic terminals. Also, pharmacological manipulations that affect presynaptic processes, such as removal of Ca^{2+} or blockade of Ca^{2+} channels, resulted in complete abolition of the synaptic response preventing further examination of this mechanism. A presynaptic action of sulfide on synaptic responses had earlier been postulated by Baldelli (1990) in the CA1-Schaffer collateral synapse of the hippocampal formation. This is further supported by neurochemical data (this thesis), where HS$^-$ is observed to alter the release of amino acid neurotransmitters. If sulfide acts at presynaptic terminals as it does at the DR cell body, the activation of conductances might alter the release of neurotransmitters.

That postsynaptic actions of sulfide contribute to the inhibition of the synaptic response is suggested by the observation that it produces a greater degree of inhibition of synaptic responses in cells that also exhibit postsynaptic responses to sulfide (≈ 80% at 200 μM) than in cells having no measurable a postsynaptic response to sulfide (≈ 20% at 200 μM). A postsynaptic mechanism by which HS$^-$ may inhibit synaptic transmission could be through the alteration in the postsynaptic conductance of the cell. HS$^-$ may accomplish this by one of two ways: indirectly through the release of transmitters which in turn activate various receptors leading to the changes in postsynaptic conductance or directly by activation of membrane channels that change the postsynaptic conductance of the cell. The observation that supramaximal concentrations of antagonists to excitatory and inhibitory amino acids, α_1-adrenergic and 5-HT_1 receptors attenuate but does not abolish the HS^- induced postsynaptic response (Fig. 28) suggests that sulfide produces its postsynaptic effect via both mechanisms. Further support for this hypothesis would be if the postsynaptic responses were reversed by the application of TTX. Another possible postsynaptic mechanism that could contribute to the synaptic inhibition would be if sulfide altered receptor-agonist interactions (such as binding affinity; K_D and receptor density; B_{max}). Irrespective of the mechanism by which HS^- produces these postsynaptic effects, the consequence of these actions would be a change in membrane conductance and the shunting and/or occlusion of conductance changes induced by synaptically released transmitters.

V. PROTECTIVE ACTIONS OF DITHIOTHREITOL (DTT) IN SULFIDE POISONING.

The role of free thiols and disulfide bridges in receptor and protein function is well studied (Blanchard et al., 1982; Brose et al., 1989; Fraser, 1989; Derkach et al., 1990; Ben-Haim et al., 1975 & 1973; Rang and Ritter, 1971). Cysteine and cystinyl thiol residues are particularly important in receptor-ligand interactions. Wood (1987) first suggested that HS^- may react with tissue cysteine and cystinyl-containing peptides to form persulfide compounds of the general formula R-S-S-H. Such an interaction, one would speculate, would lead to alteration in protein conformation and thus to a change in receptor-ligand interaction and hence receptor function. Also, other structural proteins with free thiols and disulfides will be targets of such an action resulting in functional alteration. Both these events could underly the toxicity of HS^-. If so, then protection of thiols and/or disulfides from such persulfide formation could prevent or reverse the toxic effects of HS^-.

A compound capable of protecting or rescuing thiols and disulfides, by reducing them, was first synthesized and described for this purpose by Cleland (1964) (see review by Jocelyn, 1987). This compound, dithiothreitol (DTT) or the erythro-analog dithioerythreitol (DTE) are themselves thiol compounds but with very low redox potentials (0.33 V at pH 7; and 0.366 at pH 8.1) and are therefore oxidized at the expense of the functional thiols or can rescue oxidized thiols, such as persulfides, by reducing them in a general reaction of the form:

$$\text{R-S-S-R} + \text{HS-CH}_2\text{(CHOH)}_2\text{CH}_2\text{-SH} \rightleftharpoons \text{R-SH} + \text{R-S-S-CH}_2\text{(CHOH)}_2\text{CH}_2\text{-SH} \rightarrow \text{cyclic -S-SCH}_2\text{(CHOH)}_2\text{CH}_2\text{-} + \text{R-SH.}$$

We therefore tested the hypothesis that DTT would protect or reverse the toxic effects of HS^- in rats. To ensure that DTT toxicity itself (Sax and Lewis, 1985) did not contribute to the toxic effects of HS^-, the toxicity profile of DTT was determined in rats (as there was no toxicity data available for DTT in any other animal but mice). The LD_{50} as determined by the method of Read and Munch (Turner, 1965) was approximately 200 mg/kg. The extrapolated LD_1 value (81 mg/kg) was then used for protective or treatment studies.

To examine the ability of DTT to reverse the actions of sulfide *in vivo*, DTT was administered by IP injection 30 and 60 sec after animals were treated with different doses of sulfide by the same route. In these studies, no reversal of sulfide toxicity was observed when DTT was given at any time after high concentrations of HS^- ($\geq LD_{50}$ concentration). However a significant degree of survival (- 50% reduction in mortality) was observed when rats were given a low dose of HS^- (LD_{50}) and DTT treatment commenced almost immediately (30 sec after sulfide). These observations suggest that DTT could probably reverse the actions of sulfide if animals lived long enough for it to act. This is further supported by the fact that death after sulfide poisoning occurs rapidly (within 2 min) so that at high concentrations, irreversible damage may be done before DTT administration.

To test the hypothesis that DTT, if allowed sufficient time to act, would reverse the toxic actions of sulfide *in vivo*, rats were pretreated with DTT for 20 min and then various doses of sulfide were given and a dose-lethality curve drawn. The DTT pretreatment shifted the sulfide lethality curve to the right almost doubling the estimated LD_{50} concentration of sulfide (from 15 mg/kg to 26 mg/kg; Warenycia et al., 1990). This indicates that DTT is capable of preventing or reversing sulfide toxicity *in vivo* if sufficient time exists between sulfide exposure and DTT administration. These time-dependent actions of DTT might be due to slow absorption from the site of injection, distribution to the CNS or even the rate of the chemical reaction involved. Although the toxicokinetics of DTT is unknown, its relative size suggests that it will be found to be slower than the small, simpler HS^- molecule.

To determine if DTT affected the electrophysiological actions of sulfide, the effect of DTT on a sulfide-induced membrane current in DR cells was examined. The application of 527 μM DTT (equivalent to the LD_1 concentration of 81 mg/kg) produced an inward current that increased steadily with time. The application of sulfide at various time intervals after DTT application began revealed that DTT inhibited or attenuated the HS^--induced outward current in DR cells. This inhibition was both concentration and time-dependent with significant inhibition (> 50%) occurring only after 30 min pretreatment with 527 μM DTT. 263 μM DTT (1/2LD_1 concentration) produced neither a measurable inward current nor a measurable attenuation of the sulfide-induced outward current in over 30 min of application (not shown). This delayed action of DTT is consistent with *in vivo* observations where DTT produced significant protection from sulfide actions only after 20 min pretreatment.

To determine if DTT could reverse the actions of sulfide *in vitro*, 527 μM of DTT was applied at the peak of the sulfide-induced outward current. DTT reversed this peak current to resting level and beyond, but over a relatively long time (> 4 min). This time is considered long since death due to sulfide poisoning occurs approximately 2 min after exposure. These electrophysiological actions of DTT on the sulfide induced response in the DR nucleus concur with the *in vivo* observations of their interaction. These observed effects of DTT suggest that its action may be too slow to be of any use during acute lethal exposure to sulfide. It still may be of use in "knockdown" situations where unconsciousness persists for extended periods even though spontaneous respiration returns after assisted respiration has been applied.

The above results (both *in vivo* and *in vitro*) suggest that an interaction of HS^- with thiol and disulfide bridges (formation of persulfides) may be the mechanism by which HS^- produces its toxicity. This is supported by the fact that persulfide generation or thiol oxidation are known to alter the function of several enzymes, structural proteins and receptors (Valentine et al., 1987; Derkach et al., 1990; Aizenmann et al., 1989; Ben-Haim et al., 1975 & 1973; Rang and Ritter, 1971) and DTT, a thiol protecting agent, has been shown to both liberate sulfide from poisoned rat brains (Warenycia et al., 1990) and also to reverse or protect against sulfide-induced inhibition of thiol-rich enzymes (Warenycia et al., 1989) and some receptor-mediated processes (Derkach et al., 1990). These results would suggest that a rational biochemical or chemical design of antidotes to HS^- poisoning should be geared towards more efficacious, rapidly acting thiol and disulfide protecting agents.

VI. SUGGESTIONS FOR FURTHER EXPERIMENTS.

A great deal of questions remain unanswered regarding the actions of sulfide on neurons. Although the present evidence suggests that sulfide activates a calcium-dependent K^+ conductance (gK_{Ca}), two types of this current exist that show different pharmacology, kinetics and voltage-dependence. It will be interesting to know whether a particular subtype or both are involved in the sulfide induced outward current. This can be done by application of either apamin, which blocks only SK, or charybdotoxin or low concentrations of TEA, both of which selectively block maxi-K.

Since the present evidence suggests that sulfide may produce the outward current by inducing a calcium influx, it would also be of interest to know the effects of sulfide on calcium channels or currents recorded in these neurons and/or other neurons. In pursuing the latter objective, it would be good to determine which subtype(s) of calcium channels are involved if HS^- acts on identifiable subtypes. The latter can be achieved by both biophysical manipulations and pharmacological isolation of the different calcium currents (Nowycky et al., 1985; Hille, 1991).

As sulfide is known to inhibit oxidative phosphorylation and hence ATP availability, one would hypothesize that sulfide would modulate the ATP-sensitive K^+ conductance. Although intracellular application of ATP did not appear to affect the sulfide-induced K^+ current, since the mechanism by which ATP modulates the gK_{ATP} is not properly understood, it will be useful to examine other blockers of I_{ATP} to determine whether the above observation will be confirmed. This can be done by applying sulfide in the presence of a sulphonylurea compound or other oral hypoglycaemic agent, such as tolbutamide and glyburide which block I_{ATP} (Dreyer, 1990; Hille, 1991).

To date, the mechanism(s) underlying the inward current (depolarization) induced by sulfide in both amphibian and mammalian neurons is not known. Although the present data might implicate inhibition of the Na^+/K^+ ATPase pump as contributing to this current, complete blockade of ATPase does not eliminate the inward current. Careful examination of the charge carriers of the strophanthidin-insensitive components will shed more light on the actions of sulfide on neurons. Particularly intriguing is the extremely weak voltage-dependence of the HS^--induced inward current. As it was observed that Cd^{2+} partially blocks this current while not affecting the conductance a more detailed examination of the action of Cd^{2+} is warranted.

It will also be interesting to know if cyanide, a compound with a great deal of toxic similarity to sulfide produced some of these effects of sulfide on DR or brain stem neurons. If such a study is done, it should be possible to contrast the activation of gK_{Ca} by cyanide in central neurons to those of sulfide and also to compare the central to the peripheral nerve actions of cyanide since cyanide activates this current in carotid cells only through the release of intracellular calcium (Biscoe and Duchen, 1989, Biscoe et al., 1989).

As sulfide appears to produce several different effects in DR and hippocampal neurons, it will be important to determine the action of sulfide on a few more selected brain regions and on peripheral neural mechanisms before general statements about the neurotoxicity of sulfide can be made.

Finally, for the purpose of developing affective antidotes or protective agents against sulfide poisoning, other thiol interacting compounds should be screened in laboratory animals to determine more efficacious ones for further testing. This is suggested by the fact that the action of DTT appears to be too slow to be of any use in acute intoxication.

VII. SUMMARY AND CONCLUSION

Hydrogen sulfide is an environmental toxin frequently encountered in over 70 occupational and industrial settings, including paper pulp mills, urban sewers, farming, oil and gas exploration and refining. Following intoxication, victims display signs and symptoms that implicate CNS disturbance. Victims show motor disturbance, respiratory distress and ultimately death (Burnett, 1977; Tvedt et al., 1991). Also, human survivors often display varying degrees of amnesia (Tvedt et al., 1991). The objective of this study was to examine the actions of sulfide on the nervous system to determine if sulfide induced changes in the properties and function of nerve cells that are consistent with these toxic manifestations. The study also involved tests of potential antidotal substances for sulfide poisoning. It was done using the sodium hydrosulfide salt (NaHS) which dissociates in solution to yield hydrogen sulfide anions which are the predominant toxic ions produced by hydrogen sulfide (H_2S) gas at physiological pH. The ionization equations are as follows:

$$H_2S \rightleftharpoons H^+ + HS^- \rightleftharpoons H^+ + S^{2-}$$

In addition to generating an identical toxic ion as the natural toxin and producing similar neuropathological changes, the salt also has the additional advantages of being safer to handle, allows for greater accuracy of dosage and requires no special (expensive) equipment for administration.

Since neurotransmitters play an essential role in CNS function, the total content, release and metabolism of different neurotransmitters were examined in brain regions known to control or modulate the symptoms observed during or after sulfide poisoning. These include the cortex, cerebellum, hippocampus, striatum and brain stem. Supernatants from processed rat brain samples were analyzed by HPLC using either fluorescence or electrochemical detection methods.

Acute intoxication of rats (IP injection of aqueous solutions of NaHS) increased the amounts of alanine, aspartate, GABA, glutamate, glutamine, glycine and taurine mainly in the brain stem. Only minor changes occurred in the cerebellum while no changes were observed in the amino acid levels in the other brain regions examined. Also, profound increases in serotonin (5-HT), dopamine, noradrenaline and adrenaline were observed in the brain stem after treatment with toxic amounts of sulfide. While the hippocampus and striatum showed similar increases in noradrenaline and adrenaline but not dopamine, little or no change was seen in the catecholamine levels in the cerebral cortex and cerebellum. These changes in the biogenic amine levels after treatment with sulfide may be due to inhibition of their catabolic enzyme, MAO and COMT. This is suggested by the ability of sulfide to inhibit the MAO enzyme both *in vitro* and *ex vivo*. Although COMT may play a role in this action, the effect of sulfide on this enzyme is unknown and was not examined.

Released amino acid neurotransmitters were recovered from selected brain regions by the push-pull perfusion technique and the perfusate analyzed by HPLC with fluorescence detection. NaHS was administered either by IP injection of the LD_{50} dose or by direct local perfusion of the region under study with a solution of sulfide in ACSF (3-4 μg/ml solution, equivalent to the concentration of S^{2-} in the brain after IP injection of an LD_{50} dose). While perfusates recovered after IP injection of sulfide showed little or no change in the amino acids examined, analyses of perfusates from animals which received sulfide by local perfusion revealed both immediate and delayed increases in the amounts of several neuroactive amino acids recovered in the hippocampus and striatum. However, only a delayed decrease in the level of glycine was observed in a selected brain stem nucleus (the region bordering and including the dorsal aspect of the nucleus ambiguus). The mechanism by which sulfide induces these increases is not known and can only be speculated to be via alteration in any or all of the following processes: alteration in the presynaptic release processes, inhibition of metabolic enzymes that normally contribute to the termination of the action of released transmitters and inhibition of reuptake processes that are also important in the termination of the action of released transmitters. The differences in effects between the routes of sulfide administration may be due to the well documented rapid detoxification processes that are abundant in the systemic circulation.

Since neurotransmission involves both chemical and electrical processes and neurochemical changes occur on a relatively slow time scale (seconds to minutes), the action of sulfide on the faster (msec range) neuronal electrical events were examined *in vitro* using both frog sympathetic ganglia and rat brain stem slices containing the dorsal raphe nucleus. The effects of sulfide on neuronal properties and neurotransmitter- and agonists-induced responses were examined.

In view of the neurochemical changes induced by sulfide on biogenic amine levels and the effect of sulfide on acetylcholinesterase activity (Maneckjee, 1985;

reviewed by Khan, 1989), the action of sulfide on α_2-adrenergic and both nicotinic and muscarinic cholinergic responses were examined in frog sympathetic ganglia using the sucrose-gap recording technique. Sulfide induced a reversible depolarization in these cells. This depolarization was insensitive to the removal of extracellular Na^+ or K^+ ions suggesting the depolarization was not due mainly to pump inactivation. This correlates with evidence, from rat DR neurons, that the sulfide-induced inward current was not due to inhibition of ATPase. In addition, sulfide reversibly potentiated the hyperpolarizing responses induced by adrenaline and muscarine but not the depolarizing response induced by nicotine. Since muscarine and probably adrenaline mediate slow synaptic responses while nicotine mediates fast synaptic responses in these cells, sulfide may only modulate slow synaptic responses in these cells. However, the sulfide-induced depolarization may account for the potentiation of the muscarinic and adrenergic responses. Also, sulfide appeared to modulate the electrogenic Na^+/K^+ ATPase pump in these cells. Although the presence of sulfide did not alter the K^+-activated pump dependent hyperpolarization, it produced a marked, reversible potentiation of this response after its removal.

While the above study provided preliminary evidence that sulfide produced changes in neuronal properties and modulated some but not all neurotransmitter-and agonist-induced responses, the technique could not and did not address the detailed mechanism(s) underlying those changes. As amphibians are very different from humans, it was necessary to examine the actions of sulfide on a mammalian nervous system which is more identical to that of the human. Thus, the actions of sulfide on rat neurons were examined. Because death from sulfide is thought to result from cessation of respiratory drive from the brain stem, a brain stem nucleus involved in the generation and maintenance of respiratory rhythm would have been ideal for such an investigation. However, because of the technical difficulty of intracellularly recording from cells in such nuclei, and consequently the general lack of detailed electrophysiological characterization of these cells, the dorsal raphe (DR), a pontine stem nucleus with well characterized synaptic and neuronal properties was used as a "model" brain stem nucleus to examine the actions of sulfide. This nucleus also provided an unique system in which to examine the interaction of sulfide with several neurotransmitters as it receives synaptic inputs of several pharmacological types. These studies were done using both conventional sharp microelectrode intracellular recording and the "gigaohm seal" whole-cell recording techniques (Hamill et al., 1981), in both current and voltage clamp modes.

Sulfide produced different responses in voltage clamped DR cells *in vitro*. Approximately 14% of DR cells responded to sulfide with a slow onset voltage-dependent outward current accompanied by an increase in conductance. This current reversed polarity at approximately -90 mV (E_{rev}). This E_{rev} shifted in the positive voltage direction with increasing extracellular K^+ ion concentration. However, the reversal potentials at all extracellular K^+ ion concentrations (including in normal ACSF) were more positive than the values calculated from the Nernst equation for these cells (assuming an intracellular K^+ concentration of 135 mM). The above observation, nonetheless suggest that the outward current is carried by K^+ ions and that sulfide is activating or modulating K^+ channels in these cells. The sulfide-activated outward current was partially blocked by extracellular Ba^{2+} alone, and completely eliminated by a combination of Ba^{2+} and Cs^+. The complete blockade of the outward current by Ba^{2+} and Cs^+ unmasked an inward current induced by sulfide. This underlying inward current may account for the deviation of the E_{rev} of the outward, HS^--induced current from those predicted by the Nernst equation. The outward current was also completely eliminated by extracellular cadmium (Cd^{2+}), an inorganic calcium channel blocker, which also unmasked a small inward current. This action of Cd^{2+} implies the involvement of extracellular Ca^{2+} ions in this sulfide mediated response. This was further supported by the observation that removal of extracellular Ca^{2+} also completely abolished the sulfide induced outward current. These observations therefore suggest that sulfide may be acting to activate Ca^{2+} channels leading to the influx of Ca^{2+} ions which in turn activate the outward current. Thus, the sulfide mediated K^+ current probably involves the activation of a calcium-sensitive potassium channel (gK_{Ca}). Whether one or both known subtypes of Ca^{2+}-activated K^+ channels (maxi-K or SK) are involved in this response was not examined. Furthermore, the role of intracellular Ca^{2+} ions in this response was not critically examined although the response following the removal of extracellular Ca^{2+} and the application of Cd^{2+} would suggest it contributed little, if at all, to the sulfide induced outward current.

Although the K^+ conductance induced by sulfide showed all the characteristics of a calcium-activated potassium conductance (gK_{Ca}), other K^+ conductances may contribute to this response. Because sulfide blocks oxidative phosphorylation and consequently ATP availability, sulfide would be expected to activate ATP-sensitive K^+ channels. However, intracellular application of ATP did not alter the sulfide induced outward current. Therefore, the gK_{ATP} does not appear to be involved in this response. This conclusion should however, be tempered with caution as the detailed mechanism of the K^+ channel modulation by ATP is not yet understood and the ATP levels and/or reserve in these cells are not known.

The largest proportion (43%) of DR cells responded to sulfide with a rapid onset, weakly voltage-dependent inward current similar to that unmasked by Ba^{2+} and Cs^+ in apparently pure outward current cells. This current was accompanied by little or no change in conductance. It was only partially sensitive to Cd^{2+} and the deletion of extracellular Ca^{2+} from the perfusate suggesting a role for calcium in the current. The combination of Ba^{2+} and Cs^+ did not affect it neither was the blockade of the Na^+/K^+ ATPase by strophanthidin. All these observations suggest that the inward current is probably carried by several cations including a contribution from the inhibition of the electrogenic Na^+/K^+ pump.

The third group of DR cells (30%) responded to sulfide with a biphasic response, first a rapid onset inward current that was superceded by a sustained outward current. The outward current (late phase) had characteristics similar to those recorded in the monophasic outward current responders. It was eliminated by the combination of Ba^{2+} and Cs^+, leading to a complete isolation of the initial inward current (early phase). The initial phase also had characteristics similar to those recorded in the monophasic inward current responding cells.

The above postsynaptic effects of sulfide may be due in part to an action of sulfide to elicit presynaptic neurotransmitter release. This is suggested by the attenuation of the sulfide response in two neurons by a cocktail containing supramaximal concentrations of antagonists to all known transmitters in DR. Finally, a small fraction of DR cells (13%) did not have any postsynaptic response to sulfide although the synaptic responses in these cells were sensitive to the actions of sulfide. This group probably constitutes a subpopulation of DR serotonergic cells, as they satisfied all the criteria that were used to identify all DR serotonergic cells. The presence of all these four effects of sulfide on DR serotonergic neurons suggests a greater heterogeneity in the cellular physiology of DR cells than has been previously supposed.

In most responding DR cells, the removal of sulfide was followed by a washout outward current (hyperpolarization in current clamp) presumably due to the reactivation of a previously inhibited electrogenic Na^+/K^+ ATPase pump. This hypothesis is supported by the sensitivity of the washout outward current to strophanthidin, an inhibitor of the Na^+/K^+ ATPase.

Sulfide inhibits synaptic responses, evoked by focal electrical stimulation in DR neurons. Both fast and slow synaptic responses were inhibited by toxicologically relevant concentrations of sulfide as the estimated EC_{50} values of inhibition (45-103 μM) of all the components of the complex synaptic response are within the toxic range of sulfide (LD_{50} = 15 mg/kg ≈ 80 μM). The mechanism(s) of this inhibition includes both pre- and postsynaptic processes. The presynaptic inhibition is inferred from the inhibition of the synaptic response recorded in cells with no postsynaptic changes in membrane conductance in the presence of sulfide. These included the non-responding cells and responding cells in which very low concentrations of sulfide were applied. The presynaptic mechanism(s) of sulfide action is unknown. The postsynaptic mechanism of synaptic inhibition may be via a shunting and/or occluding action of the sulfide evoked conductance change on neurotransmitter-induced responses.

If any or all of these synaptic and neuronal membrane effects of sulfide on DR neurons were to occur in other brain stem neurons, especially in the respiratory related neurons, then cessation of respiratory drive would occur rapidly leading to death. Furthermore, any or all of these actions of sulfide on other CNS neurons will lead to changes that could account for the signs and symptoms observed during or following hydrogen sulfide poisoning. These actions of sulfide on neuronal properties and functions (neurochemical and electrophysiological) are therefore consistent with the toxic neurological manifestations seen during or after hydrogen sulfide exposure.

Pretreatment with DTT at least 20 min before sulfide significantly protected rats from the actions of sulfide. However, death was too rapid for this agent to provide resuscitation when given after sulfide exposure. These *in vivo* observations were partly supported by *in vitro* electrophysiological studies in the DR where DTT significantly attenuated the sulfide-induced outward current but only after prolonged pretreatment (> 15 min). Also, DTT reversed this effect of sulfide on DR neurons but only after a relatively long period of application. It would therefore appear that interventions which actively remove or prevent access of sulfide to its interacting or binding sites (thiol and disulfide bridges) should hasten or improve recovery from sulfide poisoning. The antidotal potential of DTT in acute lethal exposure to sulfide is however limited as death occurs too rapidly for this slowly acting compound to be of benefit. However, in cases of extended unconsciousness or non-life threatening exposures, DTT may provide rapid recovery from the effects of sulfide or prevent the development of long term neurological sequelae to hydrogen sulfide poisoning.

CHAPTER V

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